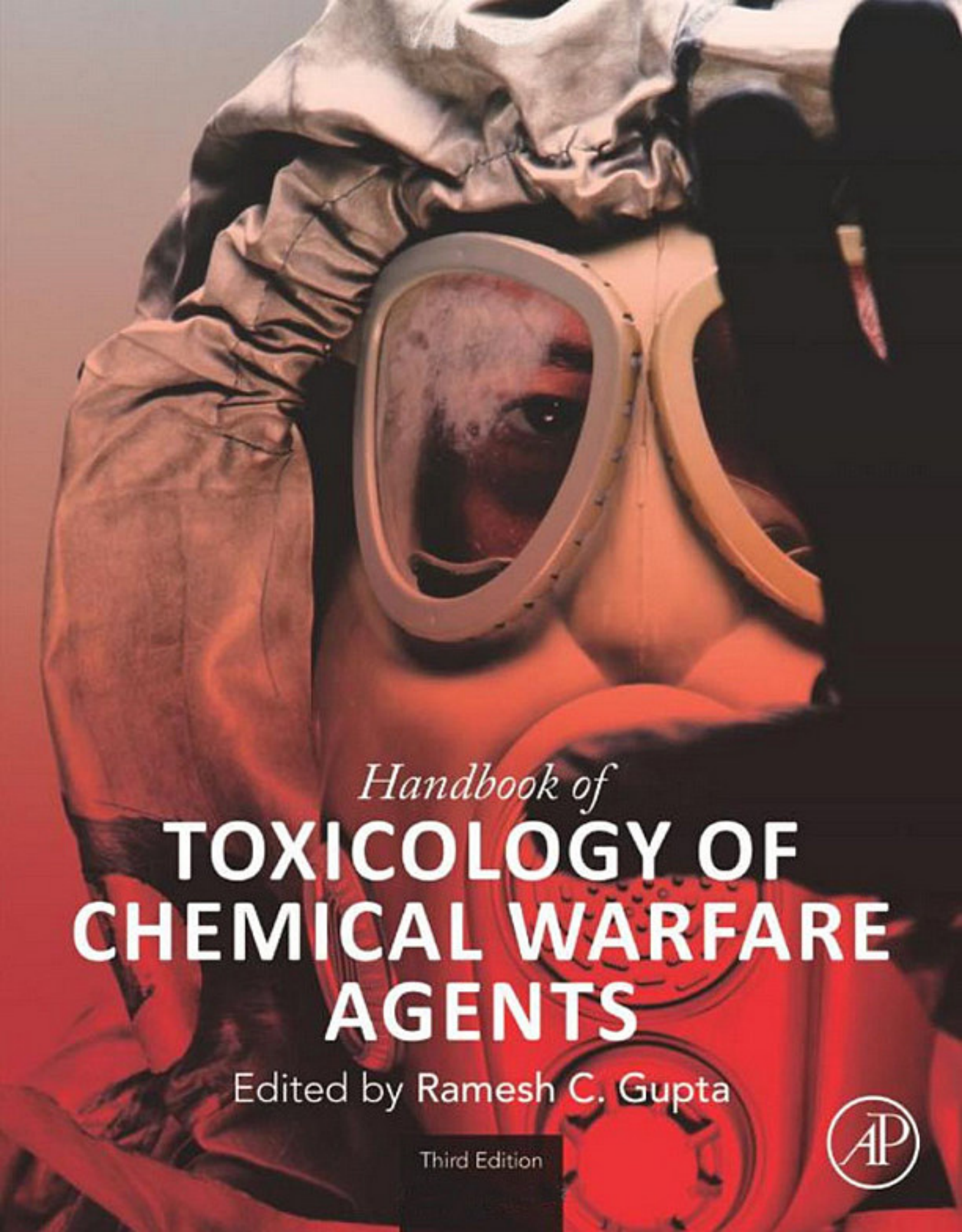

Handbook of

TOXICOLOGY OF CHEMICAL WARFARE AGENTS

Edited by Ramesh C. Gupta

Third Edition

Handbook of Toxicology of Chemical Warfare Agents

THE UNCERTAINTY OF THE DANGER BELONGS TO THE ESSENCE OF TERRORISM

Jurgen Habermas (1929—Present)

Handbook of Toxicology of Chemical Warfare Agents

Third Edition

Edited by

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Dedication

This book is dedicated to my beloved wife Denise, daughter Rekha, and parents, the late Chandra and Triveni Gupta.

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Introduction

Extremely toxic chemicals have been used for thousands of years in wars, conflicts, and extremist activities by terrorists and dictators in malicious poisonings and executions. One of the earliest forms of chemical warfare agents (CWAs) was natural toxins from plants or animals, which were used to coat arrowheads, commonly referred to as "arrow poisons." Ancient use of some CWAs and riot control agents (RCAs) dates back to the 5th century BCE during the Peloponnesian War, when the Spartans used smoke from burning coal, sulfur, and pitch to temporarily incapacitate and confuse occupants of Athenian strongholds. The Spartans also used bombs made of sulfur and pitch to overcome the enemy. The Romans used irritant clouds to drive out adversaries from hidden dwellings. In the 15th century, Leonardo da Vinci proposed the use of a powder of arsenic sulfide as a chemical weapon.

Modern use of CWAs and RCAs or incapacitating agents dates back to World War I (WWI). With advancements in science and chemistry in the 19th century, the possibility of chemical warfare increased tremendously. The first full-scale use of CWAs began in April 1915 when German troops launched a poison gas attack at Ypres, Belgium, using 168 tons of chlorine gas, killing about 5000 Allied (British, French, and Canadian) soldiers. During WWI, the deployment of CWAs, including toxic gases (chlorine, phosgene, cyanide, and mustard), irritants, and vesicants in massive quantities (about 125,000 tons), resulted in about 90,000 fatalities and 1.3 million nonfatal casualties. The majority of deaths in WWI were a result of exposure to chlorine and phosgene gases. During the Holocaust, the Nazis used carbon monoxide and the insecticide Zyklon-B, containing hydrogen cyanide, to kill several million people in extermination camps. Poison gases were also used during the Warsaw Ghetto Uprising in 1943. Again, in November 1978, religious cult leader Jim Jones murdered over 900 men, women, and children with cyanide.

Prior to, during, and after World War II, anticholinesterase organophosphate (OP) nerve agents/gases were developed in Germany, the United States, the United Kingdom, and Russia, and were produced in large volumes in many other countries. They were maximally produced and stockpiled during the "Cold War" period. These nerve agents have been used in wars and by dictators, extremists, cult leaders, and terrorist groups as chemical weapons of mass destruction (CWMD) on many occasions. In 1980, Iraq attacked Iran, employing mustard and OP nerve gases. During the period of the Iraq and Iran conflict (1980–88), Iran sustained 387 attacks and more than 100,000 troops were victims along with a large number of civilians. Thousands of these victims still suffer from long-term health effects. Shortly after the end of the Iraq–Iran war in 1988, the brutal dictator of the Iraqi regime, Saddam Hussein, used multiple CWAs against the Kurdish minorities in Halabja, killing more than 10% of the town's 50,000 residents. To date, mustards have been used in more than a dozen conflicts, killing and inflicting severe injuries in millions of military personnel and civilians.

During the Persian Gulf War, exposure to OP nerve agents occurred from the destruction of munitions containing 8.5 metric tons of sarin/cyclosarin housed in Bunker 73 at Khamisyah on March 4, 1991, and additional destruction of these nerve agents contained in rockets in a pit at Khamisyah on March 10, 1991. Although exposure levels to nerve agents were too low to produce signs of acute toxicity, military personnel serving in and around the Khamisyah area still suffer from long-term adverse health effects, most notably "Gulf War syndrome." In 1996, about 60,000 veterans of the Persian Gulf War claimed to suffer from "Gulf War syndrome" or "Gulf veterans' illnesses," possibly due to low-level exposure of nerve agents, mustard, pyridostigmine bromide, and pesticides. Exposed veterans had an increased incidence of chronic myelocytic leukemia and increased risk of brain cancer deaths compared to unexposed personnel.

In the mid-1990s, two terrorist attacks by a fanatic religious cult, Aum Shinrikyo (Supreme Truth), known as Aleph since 2000, took place in Japan (Matsumoto, 1994 and Tokyo subway, 1995). In both incidents, the OP nerve agent sarin was used as a CWA. Aum Shinrikyo in Kamikuishiki, Japan, manufactured an estimated 70 tons of sarin. Although the total fatality count involved not more than 20 civilians, injuries were observed in more than 6000 and millions were terrified. These acts of chemical terrorism were unprecedented and the impact

propagated throughout not only Japan, but the entire world. In the past few decades, many incidents have also occurred with biotoxins such as ricin and anthrax. Publicity surrounding frequent recent use due to easy access and copycat crimes increase the possibility of future use of these chemicals and biotoxins, which warrants advancement in emergency preparedness planning at the federal, state, and local government levels.

It is interesting to note that toxic chemicals have been used by governmental authorities against rebels, or civilians. In the 1920s, Britain used chemical weapons in Iraq "as an experiment" against Kurdish rebels seeking independence. Winston Churchill strongly justified the use of "poisoned gas against uncivilized tribes." The Russian OSNAZ forces used an aerosol containing fentanyl anesthetic during the Moscow theater hostage crisis in 2002. RCAs were frequently used in the US in the 1960s to disperse crowds in riot control.

Intoxications or deaths by poisoning of emperors, heads of countries, and other significant individuals have been recorded for a long time. The French Emperor Napoleon Bonaparte was poisoned with a mixture of heavy metals including arsenic and mercury. Napoleon Bonaparte died on May 5, 1821, while he was in exile on the island of St. Helena. In December 2004, during the presidential campaign, the former President of Ukraine, Viktor Yuschenko, was poisoned by a very high dose of 2,3,7,8-tetrachlorodibenzodioxin (TCDD). Ex-lieutenant of the Russian Federal Service, Alexander Litvinenko (1962−2006) died on November 23, 2006, from intoxication with polonium 210. Kim Jong-Nam, a half-brother of North Korean dictator Kim Jong-Un was poisoned with VX nerve agent at Kuala Lumpur airport in Malaysia. He died within 20 min of exposure. On March 4, 2018, the former officer of the Russian Main Intelligence Directorate, Sergey Skripal, and his daughter, Yuliya Skripal, were poisoned with Novichoks in Salisbury, United Kingdom. Following an aggressive antidotal therapy, fortunately both survived.

At present, more than 25 countries and possibly many terrorist groups possess CWAs, while many other countries and terrorist groups are seeking to obtain them, due to their easy access. Some of these agents are stockpiled in enormous quantities and their destruction and remediation are not only expensive but also associated with significant health risks. There is also the possibility of accidental release of CWAs or CWMD at their production sites, as well as during transportation, dissemination, and deployment. The intentional or accidental release of highly toxic chemicals, such as the nerve agent VX (Dugway Proving Ground, Utah, 1968), Agent Orange (Vietnam, 1961−72), PBB (Michigan, USA, 1973), dioxin (Seveso, Italy, 1976), and methyl isocyanate (Bhopal, India, 1984), has caused injuries to more than a million people, and deaths in several thousands. A 1968 accident with VX nerve gas killed more than 6000 sheep in the Skull Valley area of Utah.

After September 11, 2001, the chances are greater than ever before of the use of CWMD by extremist and terrorist groups like Al Qaeda, which presents great risks to humans, domestic animals, and wildlife in many parts of the world. On November 26, 2008, Pakistani Islamic terrorists attacked Mumbai city in India at 10 different sites, including two luxury hotels, a Jewish center, a train station, and hospitals and cafes. Approximately 200 innocent people died and about 300 people were injured by bullets and smoke. It is more likely that these terrorist groups may use toxic industrial chemicals (agents of opportunity) either as such or as a precursor for more deadly CWMD. At present, many countries have established Defense Research Institutes with two major missions: (1) to understand the toxicity profile of CWAs/CWMDs and (2) to develop strategic plans for prophylactic and therapeutic countermeasures. By the turn of the 21st century, the US established the Department of Homeland Security. Many other countries also developed similar governing branches and agencies at the state and national levels to protect people and property from terrorist attacks. Among chemical, biological, and radiological weapons, the possibility of CWMD is more likely because of their easy access and delivery system. It is important to mention that understanding the toxicity profile of CWAs/CWMD is very complex, as these chemical compounds are of a diverse nature, and, as a result, treatment becomes very difficult or in some cases impossible.

In the past, many accords, agreements, declarations, documents, protocols, and treaties have been signed at the international level to prohibit the development, production, stockpiling, deployment, and use of CWAs, yet dictators and terrorists produce and/or procure these chemicals to harm or kill enemies, create havoc, and draw national and international attention. In 1907, The Hague Convention outlawed the use of chemical weapons, yet during WWI, many countries used these chemicals. The first international accord on the banning of chemical warfare was agreed upon in Geneva in 1925. Despite the General Protocol, the Japanese used chemical warfare against China in 1930. In 1933, the Chemical Weapon Convention banned the development, possession, and use of CWAs. The document was signed and implemented by more than 100 countries. Yet, during WWI many chemicals of warfare were developed, produced, and used by several countries. In 1993, another global convention banning the production and stockpiling of CWAs was signed by over 100 countries.

The delayed health effects from CWAs used in the Iraq−Iran conflict of the 1980s, sarin subway attacks in Japan, and the First Gulf War in the 1990s are still not

well understood. Recently, the Syrian government stockpiled over 1300 metric tons of chemical agents, including sarin, VX, and sulfur mustard. In August 2013, the Syrian military repeatedly attacked civilians with chemical weapons, including sarin and chlorine. More than 1300 people died and thousands were injured. Again, on April 11−13, 2014, Syrian military forces attacked civilians in Hama province with chlorine gas, killing and injuring an unaccounted number of people. Despite warnings from many countries, the Syrian army continues to use CWAs against civilians. In the present world situation, it is highly likely that these agents will be used in wars, conflicts, terrorist attacks, and with malicious intent. In such scenarios, these extremely toxic agents continuously pose serious threats to humans, animals, and wildlife.

The first edition of this *Handbook of Toxicology of Chemical Warfare Agents* was prepared in 2009 in order to offer the most comprehensive coverage of every aspect of the deadly toxic chemicals that can be used as CWAs/CWMD. Since the publication of the first edition of this *Handbook*, concerns over the use and misuse of CWAs and BWAs have become greater than ever before. The second edition of the *Handbook of Toxicology of Chemical Warfare Agents* was published in 2015. This third edition of this *Handbook* is prepared to meet the current challenges facing academicians and lay persons alike. The format employed is user friendly and easy to understand. Standalone chapters on individual chemical and a few biological agents, target organ toxicity, biosensors and biomarkers, risks to man, animals, and wildlife, and prophylactic and therapeutic countermeasures are just a few of the many novel topics covered in this volume. The chapters are enriched with historical background as well as the latest information and up-to-date references. With 73 chapters, this book will serve as a reference source for biologists, toxicologists, pharmacologists, forensic scientists, analytical chemists, local/state/federal officials in the Department of Homeland Security, Department of Defense, Defense Research Establishments, Department of Veterans Affairs, physicians at medical and veterinary emergency care units of hospitals, poison control centers, medical and veterinary diagnostic labs, environmentalists and wildlife interest groups, researchers in the area of nuclear, chemical, and biological warfare agents, and college and university libraries.

Contributors of the chapters in this book are the most qualified scientists in their particular areas of chemical and biological warfare agents and radiation. These scientists are from around the globe and are regarded as authorities in the fields of pharmacology, toxicology, and military medicine. The editor sincerely appreciates each author for his/her dedicated hard work and invaluable contributions to this volume. The editor gratefully acknowledges Robin B. Doss and Denise M. Gupta for their technical assistance. Finally, the editor remains indebted to the editors at Elsevier (Kristi Anderson, Kattie Washington, and Kiruthika Govindaraju) for their immense contributions to this book.

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Chapter 1

History of toxicology: from killers to healers

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1.1 Introduction

The word *toxicology* comes from the Greek words *toxicon*, meaning a poison, and *logos*, meaning a scientific study. Today, the term *toxicology* refers to a scientific discipline dealing with the physical-chemical properties of toxic substances, their mechanisms of action on the body, clinical symptoms of intoxications, and the prevention and treatment of various poisonings (Klaassen, 2018). It would not be an exaggeration to claim that toxicology is almost as old as mankind. The earliest mentions of toxic substances and intoxications can be found not only in ancient scientific literature, but also in Greek myths. For example, Homer describes how Odysseus sent a warrior to Egypt to bring back traditional Egyptian poisons used for arms ammunition. In another legend, Hercules soaked his weapons in poison of the sacred Lernean hydra. Last, but not least, the myth of Helen of Troy tells how her captor died because of a wound caused by a poisoned arrow.

1.2 Ancient times

Among the oldest literary sources focused on toxicology is the Ebers Papyrus (1550 BCE) (Fig. 1.1), found in 1872 in Thebes. This 20.5 m long scroll, glued from 108 smaller sheets of papyrus, is also called "The book of preparation of remedies for all parts of the body." The oldest pharmacopoeia of the ancient Egyptians contains more than 900 prescriptions for drugs for the treatment of diseases associated with the gastrointestinal tract, respiratory tract, ear, throat, nose, eye, and skin. Such prescriptions involved substances like opium, arsenic trioxide, aconitine, cyanogenic glycosides, or a herb called Dia-Dia, currently known as mandrake (*Mandragora officinalis*, *Solanaceae*) (Hallmann-Mikołajczak, 2004). Ancient Egyptian surgeons used the juice of mandrake root for anesthesia and analgesia. Later, the art of the preparation of hypnotic and painkilling remedies isolated from the mandrake root transferred from Egypt to ancient Greece. During surgical operations, the Greeks used a sponge soaked in mandrake hot juice for anesthesia. Inhalation of the vapors of this juice resulted in a deep sleep of the patient. In the works of the Roman physician Galen, we can find passages telling about large quantities of mandrake tincture that were delivered daily to Rome. Apart from the medicinal use of mandrake, one of the Roman writers mentions mandrake wine used for war purposes, thanks to which the Carthaginians defeated the enemy. The soldiers of ancient Carthage left their camp with the mandrake wine in a conspicuous place. After returning back to the camp, they thereafter easily overpowered their sleeping enemies (Emboden, 1989; Mion, 2017).

Probably the most famous poisoning of the Hellenistic period was the execution of the Greek philosopher Socrates (470−399 BCE), who was condemned to drink the extract from hemlock (*Conium maculatum*, *Apiaceae*). His death is depicted in detail in Plato's tract *Phaedo* (Hotti and Rischer, 2017; Nepovimova and Kuca, 2019). The description of poisoning corresponds exactly to the present knowledge of coniine, the main component of hemlock.

The period of ancient Greece in world history is known for the flourishing of various scientific disciplines, including medicine. The founder of the most famous school of medicine, that was located in the Greek town of Kos, was the so-called "Father of Medicine" Hippocrates

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FIGURE 1.1 Ebers Papyrus found in 1872 in Thebes (https://commons.wikimedia.org/wiki/File:A_page_from_the_Ebers_Papyrus,_written_circa_1500_B.C._Wellcome_M0008455.jpg).

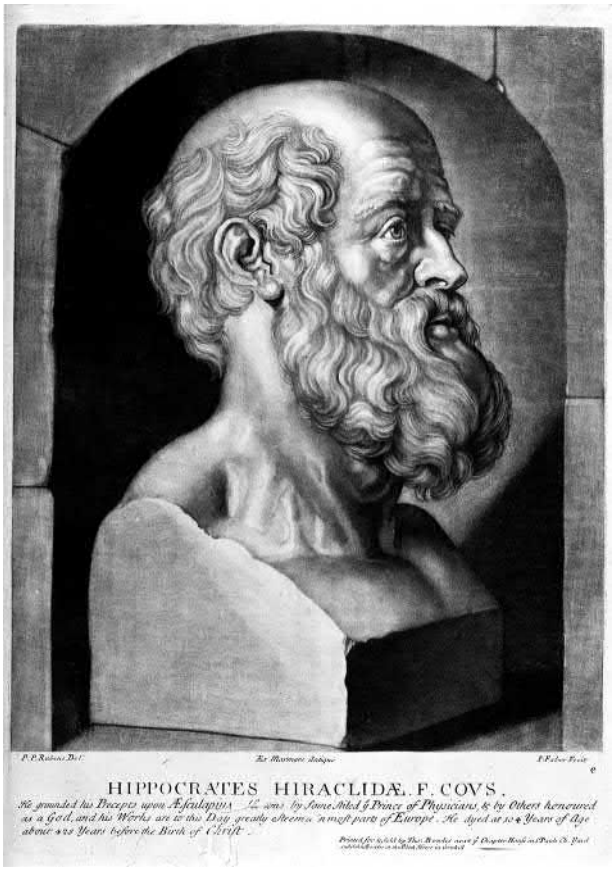

FIGURE 1.2 Father of Medicine, Hippocrates (https://commons.wikimedia.org/wiki/File:A_marble_bust_said_to_represent_Hippocrates_by_J._Faber._Wellcome_M0017663.jpg).

(460−370 BCE) (Fig. 1.2). Hippocrates rejected using poisons for removing unwanted persons. Therefore in his works, toxic substances are rarely mentioned. Some of his disciples, such as Pliny or Galen, followed the same principle, describing in their works only the antidotes. Such an informal rule has been preserved until the modern era, when young doctors, by taking the Hippocratic Oath, promise neither to administer a poison to anybody when asked to do so, nor to suggest such a course (Emery, 2013).

The ancient scientists abounded in a deep knowledge of various poisons. Usually they gained such knowledge from the observation of accidental poisonings, as well as from intentional exposure to poisons. In contrast to Eastern countries, in ancient Greece and subsequently Rome, toxic substances were quite often used as a means of killing convicts. Thus the ancient Greek poet and physician Nikander of Kolophon in his poem *Theriaca* describes clinical symptoms of intoxications by various animal toxins. In Nikander's further work *Alexipharmaca* that has survived to the present time, we find descriptions of the characteristics of plant poisons as well as methods for their treatment. As very effective therapeutic approaches he recommended invoking vomiting by drinking warm flaxseed oil or irritating the throat by simple devices made from paper or bird feathers. The majority of the knowledge reported by Nikander was based on his own experiments on convicted criminals. In addition, Nikander was by all accounts the first to describe the signs of lead poisoning (Clauss, 2012). In ancient Rome, lead was widely used in everyday life. For example, lead plates were added to wine to improve its quality. At that time, lead was very expensive and only rich people could afford it. Therefore it is not surprising that chronic lead poisoning became a scourge of the ancient Roman aristocracy (Hernberg, 2000).

From the point of view of the history of toxicology as a medical discipline, not only were poisoners and crimes committed by means of poisons important, but also searching for potent antidotes. Especially in ancient times, there was an obsession to discover a universal antidote, able to protect against most, if not all, poisons. Quite instructive is the story of King Mithridates VI of Pontus (132−63 BCE) (Fig. 1.3). Mithridates was terribly afraid of poisons, therefore, he began to study toxicology in depth—he observed the effects of various poisons on people (mostly convicts or slaves), designed antidotes, and subsequently tested their efficacy on the same groups of people. Finally, he managed to prepare a universal antidote consisting of 36 components. Such

FIGURE 1.3 King Mithridates VI of Pontus (https://commons.wikimedia.org/wiki/File:Mithridates_VI_Louvre_white_background.jpg).

an antidote even received a special designation in the Roman Pharmacopoeia—*mithridaticum.* The reputation of this antidote was excellent. It was even considered the best antidote of those times, capable of preventing the actions of aconitine, snake, scorpion, or spider toxins, etc. King Mithridates believed in his recipe so much that he decided to take this remedy daily. Acquired resistance, however, played a crucial role in his life. In old age, Mithridates attempted to commit suicide by taking a large dose of poison, but survived. Therefore he was forced to use other means (a sword) to finish this act. Based on this legend, the term *mithridatism* has been adopted into the modern toxicology indicating the increased resistance of an individual to poisons (Griffin, 1995; Valle et al., 2012, 2009).

The last wife of the Roman Emperor Claudius (10 BCE−CE 54) was his niece Agrippina the Younger (CE 15−59) (Fig. 1.4). Soon after their marriage, she decided to get rid of her husband as well as his first-born son Britannicus to make her own son, Nero, the emperor (Aveline, 2004). First, she poisoned Claudius using the toxin muscarine present in toxic mushrooms, fly agaric (*Amanita muscaria*, *Amanitaceae*), in one of his meals. The Emperor's physician, Aesculapius, tried to evoke vomiting in Claudius. However, Agrippina foresaw such a turn of events and had prepared in advance a poisoned feather. This feather was in all probability the product of the famous ancient poisoner—Locusta (Marmion and Wiedemann, 2002). After Claudius' death, Nero (CE 37−68) (Fig. 1.4) became the Emperor of Rome. Despite this, Nero's stepbrother Britannicus still constituted a threat to him (Shotter, 2008). Similarly to his mother Agrippina, Nero also asked for the help of the poisoner Locusta. In this case, she gave him a poison that was added to Britannicus' wine. After removal of his competitor, 17-year-old Nero became the only possible Emperor of Rome. Thus he decided to reward Locusta by an extraordinary right—to educate her own students. This story was one of many examples in world history where poisons were used for criminal purposes. Therefore in 81 BCE, the Roman dictator Sulla was forced to pass a special law ordering punishment, including the death penalty, for those who used poisonous substances with criminal intent (Telford, 2014).

Dioscorides (CE 40−90), the physician of the Roman Emperor Nero, in his tract *De Materia Medica* (Fig. 1.5) classified poisons based on their origin to plant, animal, and mineral. Additionally, in *De Materia Medica* we may find the methods of identification of several poisons. Such identification occurred in the scientific literature for the first time. For the next 15 centuries, Dioscorides' work was considered the "Holy Bible" of toxicology (Staub et al., 2016).

1.3 The Middle Ages

In medieval Europe, poisons were freely available in pharmacies. The first attempt to stop such a trade in poisons was made in Italy. In 1365 in Siena, apothecaries were forbidden from selling arsenic and mercury to people unknown to them. In France, a ban on toxic substances was issued in 1662, whereas in Russia this took place only in 1773 (Nepovimova and Kuca, 2019). Despite these restrictions, the question of searching for novel more potent poisons and corresponding antidotes remained relevant.

In Europe, the search for novel antidotes as well as their use in the prevention and treatment of poisoning continued until the beginning of the 18th century, and in Turkey until the beginning of the 20th century. The ancient works of Galen *De Theriaca, ad Pisonem*, *De Usu Thericae, ad Pamphilianum*, and *De Antidotis*, mainly inspired by the achievements of King Mithridates, were within the period of the Renaissance and the Middle Ages enriched with the knowledge of the Jewish physician and philosopher Moses Maimonides (1135−1204) (Fig. 1.6). His tract focused on poisons and antidotes and was published in Arabic in Cordoba (Spain) in 1198 (Rosner, 2000). This literary work constitutes a noticeable milestone in the history of toxicology, outlining the 1000-year

FIGURE 1.4 Agrippina the Younger and her son Nero (https://commons.wikimedia.org/wiki/File:Ner%C3%B3n_y_Agripina.jpg).

experience of treating various poisonings and also described the clinical picture of intoxication by poisons that were previously unknown. The first part of the tract describes intoxications as well as poisons of animal origin (bites by enraged dogs, wasps, snakes, spiders, scorpions, and other animals). As historically the first attempt, Maimonides distinguished the neurotoxic and hematotoxic symptoms of intoxication. In the second part, he focused on mineral and plant poisons. For instance, in the case of *Atropa belladonna* (*Solanaceae*) intoxication, Maimonides reported skin redness and some kind of "excitement" of the patient. As a therapeutic tool he recommended vomiting evoked by warm milk, vegetable oil, etc. (Rosner, 1968).

None of the noble families left such a significant imprint in the history of Italy and the whole world as the Spanish "holy family" of Borgia that was sadly famous for numerous murders committed by means of poisons. These Spaniards twice occupied the throne of Saint Peter—firstly as Pope Callixtus III and subsequently as Pope Alexander VI (Hibbert, 2009). "Cantarella" was the name of the poison used by the Borgias. Allegedly, Cesare Borgia (1474−1507) (Fig. 1.7), the son of the Pope Alexander VI (1431−1503) (Fig. 1.7), received the recipe for this poison from his mother. Apparently, the mystic poison contained arsenic, salts of copper, and phosphorus. According to the literary sources, the papal alchemists prepared such toxic mixtures that a drop was enough to kill a bull. Not only the poison, but also the tools containing such poison, were unique. Cesare Borgia was the owner of a ring with a huge ruby that bore the name the "Flame of Borgia." Several times he pronounced that this ring had repeatedly saved his life. Presumably, under the gemstone there was a skillfully made secret container with a poison. Cesare poured this poison into the drink of those who dared to encroach on

FIGURE 1.5 A page from Dioscorides' work *De Materia Medica* (https://en.wikipedia.org/wiki/De_Materia_Medica#/media/File:NaplesDioscuridesMandrake.jpg).

his life. The Pope himself also had a gold ring with a secret. In the process of shaking hands, a small thorn appeared on the inner side of the ring which slightly scratched the skin of the sentenced person and released a deadly drop of poison (Poole, 2010). Finally, destiny punished Pope Alexander VI, who accidentally drank poisoned wine that was intended for his victim (Hibbert, 2009).

Despite the wide use of poisons within the struggle for power, the development of toxicology in European countries in the Middle Ages was significantly hampered by the influence of religious ideologies. Medieval monks followed the principle "Like is cured by like" (*Similia similibus curantur*) (Zebroski, 2015). The exception was Swiss physician, alchemist, botanist, astrologer, and occultist of the era of the German Renaissance, Philippus Aureolus Theophrastus Bombastus von Hohenheim (1492−1541), also known as Paracelsus (Fig. 1.8). He chose this pseudonym for himself and it means "more than Celsus." Aulus Cornelius Celsus was a Roman naturalist, living more than one and half thousand years before Paracelsus (Grell, 1998). Paracelsus' groundbreaking contribution to life sciences consisted mainly in the interconnection between chemistry and medicine. Therefore it is not surprising that his life credo was: "The real purpose of chemistry is not to make gold, but to make remedies!" Paracelsus has been also considered the Father of Toxicology, since in one of his books he stated: "Dose makes the poison" (*Dosis facit venenum*). Thus substances that are taken to be toxic could be harmless in small doses, whereas normally harmless substances could be fatal if consumed excessively. This postulate still belongs among the basic pillars of modern toxicology. He was also known for his revolutionary views on the observation

of nature and man, created by himself instead of simply quoting ancient texts. Last but not least, he gave the designation to the chemical element zinc and noted that certain diseases stem from the mind of the patient (Paracelsus, 1999).

With regard to poisonings, medieval Italy and later France were considered the most powerful countries in the world. The French Queen, Catherine de' Medici (1519–89) (Fig. 1.9), also known as the Queen-Poisoner, perfectly mastered the Italian technique of poisoning to achieve her intended political goals (Kruse, 2003). Alexandre Dumas, in his historical novel "Queen Margot," wrote that Queen Catherine was involved in the death of her political rival Jeanne d'Albret by giving her an insidious present—poisoned gloves (Dumas, 1994). Within the same novel, Dumas also describes the fatal mistake of the Queen, who at the end of her life decided to remove the son of poisoned Jeanne d'Albret—Henry. She commanded he be given a poisoned book dealing with the art of hunting. Unfortunately, this book got into the wrong hands, to her own son King Charles IX. Apart from removing the competitors within the battle for the royal throne, Queen Catherine was also known for experiments with various toxic mixtures that she conducted on poor and sick people. Catherine de' Medici carefully reported each experiment, recording the velocity of the toxic response (onset of the

FIGURE 1.6 Jewish physician and philosopher Moses Maimonides (https://he.wikipedia.org/wiki/%D7%A7%D7%95%D7%91%D7%A5:Maimonides-2.jpg).

FIGURE 1.8 Philippus Aureolus Theophrastus Bombastus von Hohenheim, also known as Paracelsus (https://commons.wikimedia.org/wiki/File:Aureolus_Theophrastus_Bombastus_von_Hohenheim_(Paracelsus)._Wellcome_V0004452.jpg).

FIGURE 1.7 The Pope Alexander VI (right) and his son Cesare Borgia (left) (https://www.flickr.com/photos/hinkelstone/38111166521; https://www.flickr.com/photos/eriktorner/32671151065).

FIGURE 1.9 French Queen Catherine de' Medici (https://commons.wikimedia.org/wiki/File:Catherine-de-medici.jpg).

FIGURE 1.10 French poisoner Catherine Monvoisin (https://fr.wikipedia.org/wiki/Fichier:Catherine_Deshayes_(Monvoisin,_dite_%C2%ABLa_Voisin%C2%BB)_1680.jpg).

toxic effect), the efficacy of the toxic mixture, the strength of the toxic effect in various parts of the body (organ specificity, site of action), and the clinical picture of intoxication. Thus despite the poor reputation of the Queen-Poisoner, she can be considered the first experimental toxicologist in history (Whyte, 2001).

The development of industry in the 16th century gave rise to several highly specialized works dealing with occupational diseases. In 1556 Georgius Agricola (1494–1555), a German doctor and metallurgist, in his work "On Mining and Metallurgy" described severe occupational diseases of miners (Weber, 2002). The first real systematic contribution to occupational toxicology was made by Italian physician Bernardino Ramazzini (1633–1714). In his work "Diseases of the workers," published in 1700, where he for the first time described the diseases of workers in almost 70 professions, such as miners, gilders, chemists, plasterers, blacksmiths, etc. (Dhungat, 2017).

The Golden Age of King Louis XIV of France was not associated just with the development of the country, but also with several famous cases of poisoners—Marquis de Brinvilliers, Catherine Monvoisin, and others. Catherine Monvoisin (1640–80) (Fig. 1.10) was among the most popular poison suppliers of that time. A frequent client of Madame Monvoisin was also a hot favorite of the Sun King—Marquis de Montespan. Due to the fact that poisons were perceived as the simplest means of solving problems among the aristocratic families, King Louis XIV issued a special law, where the definition of poison was given as: "Everything that can cause a rapid death or slowly destroy human health, regardless of the fact whether it is a simple or complex substance, must be considered as a poison." To complete the story of French poisoners, Marquis de Brinvilliers, Catherine Monvoisin, and their associates were executed. Marquis de Montespan, a mother of eight illegitimate children of Louis XIV, was sent to exile in the Netherlands (Somerset, 2004).

In the late 17th–early 18th centuries, Neapolitan poisoner Teophania, more commonly known as Tophana, operated in Europe. Apparently, this Italian was responsible for the deaths of more than 600 people. Tophana was an inventor of an original product called *Aqua Tophana*. Aqua Tophana had a water-like, odorless, and colorless consistency. Allegedly, five or six drops of this magical water was enough to kill a man. The onset of the toxic effect was gradual—painless, without any sign of fever or inflammation. Death occurred due to weakness, loss of appetite, and incessant thirst. Among the most frequent customers of Aqua Tophana were women who desired to get rid of their husbands. The exact content of Aqua

Tophana remains unknown. According to one source, it was made of arsenic acid with an addition of *Herba cymbalariae* (*Scrophulariaceae*). Other sources claim that the main component of Aqua Tophana was lead acetate solution. Seniora Tophana was eventually sentenced to death and in 1709 burnt to death (Nepovimova and Kuca, 2019; Wexler, 2017).

FIGURE 1.11 The founder of toxicology Mathieu Orfila (https://commons.wikimedia.org/wiki/File:Pierre_Matthieu_Joseph_Bonaventure_Orfila._Lithograph_by_Z._Wellcome_V0004368.jpg).

At the beginning of the 19th century, the most prominent figure in toxicology was considered to be the Spanish physician Mathieu Orfila (1787–1853) (Fig. 1.11). He was the first to separate toxicology from pharmacology, clinical and forensic medicine, giving toxicology the status of an independent scientific discipline. At the age of 27, Orfila wrote a book "Treatise on poisonings," that was later published in five editions. Several years later, another work by Orfila's "A treatise on the remedies to be employed in cases of poisoning and apparent death: including the means of detecting poisons, of distinguishing real from apparent death, and of ascertaining the adulteration of wines" met with great interest from the scientific community. In his writings, the Spanish physician classified all known toxic substances, described the clinical picture of intoxications typical for various classes of poisons, and also recommended chemical methods for poison identification in biological matrices (Myers, 1961). Based on his works, it became obligatory to conduct a forensic chemical analysis for official confirmation of poisoning as the cause of the death. In addition, Mathieu Orfila gave the most general definition of poison that remains widely used "Poison is a substance, that by coming in contact with a living organism in a small amount, destroys its health and subsequently life" (Hadengue, 1987).

The 1850s could be characterized as the time of the formation of modern toxicology. The decisive influence belonged to the successes achieved in analytical chemistry and experimental analysis that won its place in theoretical medicine (Oser, 1987). The fundamental works of French scientists Francois Magendie (1783–1855) (Fig. 1.12) and his student Claude Bernard (1813–78) (Fig. 1.12) dealing with the mechanism of action of strychnine, cyanide, curare, carbon monoxide, and other poisons, served to strengthen the role of toxicology among the other scientific disciplines (Bloch, 1989). Numerous methods of

FIGURE 1.12 Francois Magendie (left) and his student Claude Bernard (right) (https://commons.wikimedia.org/wiki/File:Fran%C3%A7ois_Magendie._Lithograph_by_N._E._Maurin._Wellcome_V0003781.jpg; https://commons.wikimedia.org/wiki/File:Portrait_of_Claude_Bernard_(1813-1878),_French_physiologist_Wellcome_M0000114.jpg).

particular physiological function evaluation, such as respiration and neuromuscular conduction, proposed by Claude Bernard were preserved in experimental practice for more than 100 years. Claude Bernard was also an author of the brilliant idea that toxic substances can serve as an excellent tool in physiology research. He said: "These substances could be considered as real life reagents that are carried by the blood stream to all points of the body, act on some tissues and finally lead to the death. The mechanism of death points to the physiological role of particular tissues on which they act." This finding became a significant milestone in general physiology (Breathnach, 2014). Additionally, within his experiments with curare, Claude Bernard revealed that this poison paralyzes voluntary muscles with no effect on impulse conduction in the motor nerves as well as on contractility of the muscles. This observation led to discovery of the special sensitivity of the neuromuscular junction to curare. Several years later, these investigations served as a strong argument for the development of a theory of the neurochemical basis of excitatory transmission within the nervous system (Gomes et al., 2014).

For almost two centuries after the death of the French Emperor Napoleon Bonaparte (1769–1821) (Fig. 1.13), his demise has remained a hot topic, and the scientists continue to investigate this case. After he was sent into exile in 1820, the health status of the Corsician sharply deteriorated. Throughout his stay on the island of St. Helen, he complained about severe stomachache, weakness, and frequent attacks of nausea. Finally, on May 5, 1821, he died. According to the findings of an international group of scientists, Napoleon Bonaparte passed away due to progressive stomach cancer with metastases in the lymph nodes (Leys, 2015). However, according to conspirologists, the symptoms of the ex-Emperor's death more resemble arsenic poisoning. Moreover, recent analysis of his hair has shown an almost 40-fold increase in arsenic concentration in comparison to normal people of that time. Several theories have been formulated, explaining how Napoleon Bonaparte could have been poisoned. Among the most curious being poisonous wallpaper, or the theory claiming that Napoleon was poisoned by a mixture of heavy metals (Hindmarsh and Corso, 1998). The first hypothesis builds on the fact that adding green arsenic-based pigment into wallpaper was quite common at that time. From a chemical point of view this pigment, called Paris Green, was copper(II) acetoarsenite. The humid weather of the island promoted the proliferation of microscopic fungi which could convert inorganic arsenic in Paris Green to an organic form. It is widely recognized that organic forms of heavy metals more easily cross the biological barriers compared to their corresponding inorganic salts. Therefore this theory assumes that Napoleon was intoxicated in particular by these organic forms of

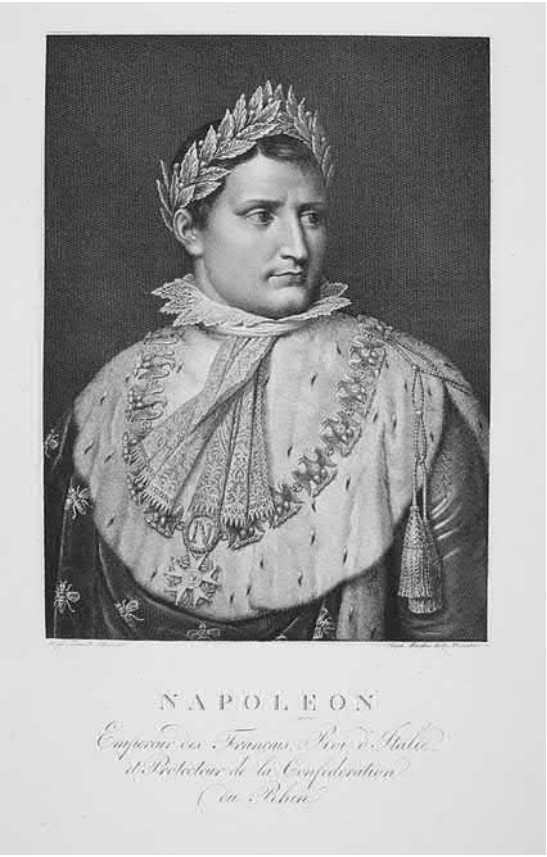

FIGURE 1.13 French Emperor Napoleon Bonaparte (https://thorvaldsensmuseum.dk/en/collections/work/E876/zoom).

arsenic. The latter theory already assumes foreign blame. According to this hypothesis, low doses of arsenic were added to Napoleon's food and drinks. The clinical picture of chronic intoxication with arsenic usually manifests as severe pain in the stomach. To relieve vomiting in Napoleon, the doctors administered him potassium tartarate of antimony. In addition to this remedy, his physicians prescribed him calomel and orgeat to combat constipation and thirst, which represent other characteristic symptoms of arsenic intoxication. The main component of orgeat is bitter almond oil, whereas calomel is a trivial name for mercury dichloride (Hg_2Cl_2). Hydrolyses of orgeat to hydrocyanic acid in the acidic gastric environment, together with calomel, gives rise to mercury cyanide. Therefore either arsenic, hydrocyanic acid, antimony, mercury cyanide, or a mixture of some or all of them could be one of the causes of Napoleon's death (Mari et al., 2004).

1.4 The modern era

The Industrial Revolution in the middle of the 19th century allowed the synthesis of natural toxins in unlimited

quantity. Moreover, novel entities derived from natural compounds were prepared. Due to all the above-mentioned events, poisons were gradually losing their mystery (Nepovimova and Kuca, 2019). At the beginning of the 20th century, the development of toxicology was strongly influenced by progress in the chemical industry. From the perspective of chemical production, Germany was among the most developed countries. Within several branches of the chemical industry, German chemists even maintained a monopoly position, for example, in dye production. One of the most famous German chemists of the time was Fritz Haber (1868–1934) (Fig. 1.14), who discovered a method of ammonia synthesis from atmospheric nitrogen. Such an invention was of high importance for the large-scale synthesis of fertilizers and explosives. Therefore in 1918, Fritz Haber was awarded the Nobel Prize (Manchester, 2002). In the history of toxicology, F. Haber is better known for another reason, he is called the "Father of Chemical Weapons" due to his longlasting reasearch in the field of weaponization of chlorine and other toxic gases in World War I (WWI). In addition, it was Haber's suggestion to use chlorine in the first chemical attack by Germans against British/French troops on April 22, 1915, near the town of Ypres (Belgium) (Charles, 2005). Subsequently, the Allies (France, Great Britain, United States, and Russia) also started to use chemicals for military purposes. During the 4 years of WWI (1914–18), about 1.3 million people were affected by chemical weapons on both sides of the conflict, of which more than 100,000 died (Tucker, 2006).

FIGURE 1.14 The "Father of Chemical Weapons" Fritz Haber (https://commons.wikimedia.org/wiki/File:Fritz_haber_1929_PI_29-C-0097.jpg).

On September 7, 1978, Bulgarian dissident Georgi Markov (1929–78), after an evening broadcast on the BBC, went around a crowded bus stop on the Waterloo Bridge in London and suddenly felt a slight sting in his leg. Looking around, the Bulgarian noticed a man picking up an umbrella from the ground. The stranger spoke with a strong accent, apologizing, and then caught a taxi and left. Due to a high fever, acute stomachache, and severe diarrhea, Markov was hospitalized that night, and a few days later he died. Fortunately, he managed to talk about the incident with the umbrella. Doctors, who performed an autopsy, found a small iridium-platinum capsule in the leg of the dissident. According to the findings of further investigations, this capsule with a diameter of less than 2 mm was filled with ricin (Crompton and Gall, 1980). Ricin is a plant toxin obtainable from the castor bean (*Ricinus communis*, *Euphorbiaceae*). By all accounts, Georgi Markov was shot by the Bulgarian special services because of his active criticism of the communist regime of Todor Zhivkov (Papaloucas et al., 2008). The killing device was an umbrella endowed with a hidden sting that shot small capsules filled with toxic ricin.

On March 20, 1995, the nerve agent sarin was used at several subway stations in Tokyo (Japan). About 10,000 people were affected, with 5000 seriously intoxicated and 12 people died. Sarin was used by the terrorists of the sect Aum Shinrikyo. They stored the nerve agent in plastic bags, which were subsequently punctured by an umbrella with a sharpened tip. The terrorists managed to puncture 10 of 11 bags. Fortunately, due to the low purity of the sarin (approximetely 30%) and the subway ventilation system, the loss of life was not as high as it might have been (Okumura et al., 2005). The question that still needs to be answered is: "Why did the sect select sarin?" There are plenty of possibilities why: (1) inspiration from the Gulf War; (2) simple synthesis; (3) starting compounds availability; and (4) low production costs (Nozaki and Aikawa, 1995). Many sect members who participated in the sarin production process claimed that they were unaware of its toxic effects. However, the handbook "Magic song of sarin," found in one of the buildings used by Aum Shinrikyo, apart from the instructions of how to synthesize the nerve agent, gave a description of its lethal effects. Therefore it was obvious that the members of the sect had lied (Kimura, 2002). The terrorist use of sarin in the Tokyo subway pointed out the serious risk of misuse of chemical warfare agents for nonmilitary purposes and highlighted the need for the development of appropriate protection including antidotal therapy.

The former President of Ukraine, Viktor Yushchenko (born 1954) (Fig. 1.15), was intoxicated by a very high dose of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in

FIGURE 1.15 Former Ukrainian President Viktor Yushchenko (https://sk.wikipedia.org/wiki/S%C3%BAbor:Viktor_Yushchenko_2006.jpg).

December, 2004. This terrible act happened during the presidental campaign. Detailed analysis revealed that Yushchenko's body contained the second highest concentration of TCDD ever detected in a human (Sorg et al., 2009). The main symptom that he suffered from was chloracne, which significantly deformed the skin on his face (Saurat et al., 2012). The same substance intoxicated thousands of Vietnamese during the war in Vietnam (1955−75).

Ex-lieutenant of the Russian Federal Service (FSB), Alexander Litvinenko (1962−2006), died in November 2006 because of intoxication with a radioactive compound. The findings of the investigators pointed to the fact that this act happened in the hotel Millennium (London, United Kingdom) during a meeting with former colleagues. Soon after the intoxication, Litvinenko's health deteriorated and, despite countless attempts by toxicologists to combat the disease, he continued to decline. On November 23, 2006, Alexander Litvinenko died (McFee and Leikin, 2009). The autopsy revealed that the cause of death was radioactive polonium 210. Among several possibilities, it was proposed that polonium had entered Litvinenko's body in tea. The ex-lieutenant died of acute heart failure. Allegedly, less than one gram of polonium 210 is enough to kill a person. After entering the body, its action is almost imposible to stop. Finally, the alpha particles of this silver powder attack the liver, kidneys, and bone marrow, resulting in gradual failure of vital organs (Harrison et al., 2007, 2017).

On February 13, 2017, a half-brother of the North Korean dictator Kim Jong-Un was poisoned. According to a video recording, Kim Jong-Nam (1971−2017) was attacked by two women at the airport in Kuala Lumpur, Malaysia. Twenty minutes later he died. His autopsy disclosed traces of an extemely toxic compound belonging to the V-group of nerve agents. Allegedly, the V-agent was in a binary form since neither of the offenders manifested any sign of intoxication (Paddock and Sang-Hun, 2017). It is highly likely that each woman was bearing a particular precursor of the binary weapon. By touching Kim Jong-Nam on the face the two precursors reacted in situ, resulting in the formation of V-agent.

On March 4, 2018, Sergey Skripal and his daughter Yuliya were intoxicated in the city of Salisbury (Great Britain). The former officer of the GRU (Main Intelligence Directorate) Sergey Skripal (*1951) had dual citizenship of Russia and Great Britain. Skripal's daughter Yuliya, a Russian citizen, had flown to London to visit her father who worked for the British Special Services. Soon afther the poisoning by a nerve agent, they were hospitalized in an uncounscious state. On April 10, 2018, Yuliya was discharged from the local hospital and taken to a hospital at a British military base. On May 18, 2019, Sergey Skripal was also discharged. British experts from Porton Down science park determined that the poison used belonged to the most recent group of nerve agents called "Novichoks," in particular, it was compound A-234. However, the scientists did not manage to reveal the origin of the compound. Later, the findings gained by British experts were confirmed by the Organization for the Prohibition of Chemical Weapons (OPCW) (Vale et al., 2018). The Novichok nerve agents were developed in the 1970s by the USSR as a reaction to the British/American invention of the VX agent. The Western world learned about the existence of the Novichoks in 1992 from a defectors who emigrated to the United States. According to the available data, these substances exert a higher or at least similar toxicity to the VX agent (Franca et al., 2019; Nepovimova and Kuca, 2018). The UK government accused Russia of an attempt to kill Sergey Skripal as well as being in violation of the Chemical Weapons Convention. Moscow categorically rejected such accusations and stated that the poisoning could have been organized by the Special Services of the United Kingdom or the United States.

1.5 Concluding remarks and future directions

This chapter points out that removal of unwanted persons via poisons was known in antiquity and is still very

popular at the present time. Almost until the 18th century, poisons were strongly associated with mystery, black magic, and occultism. Only with progress in the life sciences, including toxicology, have poisons started to be perceived differently. Such progress was accompanied by achievements in the fields of analysis and detection, chemical synthesis of novel toxic substances, and corresponding antidotes, and also the inventions of refined devices, such as in the case of the Georgi Markov assassination. Today, the most experienced toxicologists do not work in the academic field, but rather for secret services and/or military organizations. The intent for which novel poisons are prepared has also changed from mere removal of specific enemies to mass intimidation, terrorism, and false accusation. However, regardless of the intention, it is clear that toxicology as a scientific discipline will never die as new and more toxic compounds are being developing continuously.

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Chapter 2

Historical perspective of chemical warfare agents*

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2.1 Introduction

The employment of chemicals in war has a long history (Silvagni et al., 2002; Romano et al., 2008). Just as the use of chemicals brought about tremendous advances in society, the concept of using chemicals to help win wars has been pursued for centuries (Joy, 1997; Smart, 1997). There are many examples of the exploitation of chemicals in warfare and conflict dating back to ancient times. Primitive humans may have been the first to use chemical compounds in both hunting and battle scenarios. The use of smoke from fires to drive animals or adversaries from caves may have been the earliest use of chemical weapons. Natural compounds derived from plants, insects, or animals that were observed to cause sickness or death were likely used by our distant ancestors in attempts to gain or maintain superiority (Hammond, 1994). Natural toxins from plants or animals on arrowheads, as well as the poisoning of water or food, could increase casualties and cause fear in opposing military forces or civilian populations. These early uses of chemicals paved the way for more lethal chemical weapons. For example, in the 4th century BCE, smoke containing sulfur was used in the war between Sparta and Athens (Joy, 1997). Chinese manuscripts indicate arsenic-based compounds were used in battle (Joy, 1997). A few hundred years later, toxic smoke was employed by the Romans in Spain (Coleman, 2005). During the second siege of Constantinople, the Byzantine emperor Leo III used "Greek fire" in his quest for military victory (Coleman, 2005). During the ensuing years, there were many instances of the limited and attempted use of chemicals and toxins on the battlefield. Many of these examples may have been influenced by the intentional poisonings occurring in civilian settings (Joy, 1997; Smart, 1997; Newmark, 2004; Coleman, 2005). The earliest known treaty to ban poisons in warfare was signed between the French and Germans in the 17th century (Smart, 1997). In the siege of Groningen, European armies employed incendiary devices to release belladonna, sulfur, and other compounds. This led to the Strasbourg Agreement in 1675, which prohibited poison bullets (Smart, 1997; Coleman, 2005).

As science and chemistry advanced in the 19th century, the possibilities of chemical warfare increased exponentially. Advancements were made in industrial applications of sulfur, cyanide, and chlorine (Joy, 1997). In addition, the concept of delivering chemicals via projectiles was introduced. During the Crimean War, the British refused to use cyanide-based artillery shells against the Russians on the grounds that it was a "bad mode of warfare" (Smart, 1997). This was an early example of the ethical questions surrounding chemical use in warfare that continued into the 20th century (Vedder and Walton, 1925). During the US Civil War, both the Northern and Southern armies seriously considered using various chemicals in their pursuit of operational victories (Smart, 1997). Early attempts at international treaties met with mixed results. The United States prohibited any use of poison during the Civil War. The Brussels Convention on the Law and Customs of War of 1874 prohibited poisons or poison-related arms (Smart, 1997). The first Peace Conference at the Hague prohibited projectiles filled with asphyxiating or deleterious gases (Smart, 1997).

* The opinions and assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the employers of the authors.

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The employment of chemicals as asphyxiating warfare agents was vigorously discussed there (Joy, 1997). However, some countries, including the United States, were not signatories to this agreement. Arguments again were made against chemicals based on moral grounds. However, counterarguments were made based on the assumption that chemicals lead to death without suffering (Vedder and Walton, 1925; Joy, 1997; Coleman, 2005). Individuals who advocated chemicals did not view their use as an unfair advantage; rather, it was just one of a series of technological advances which, if mastered, could provide strategic, operational, and tactical advantages on the battlefield. The second Peace Conference at the Hague, held 8 years later, prohibited both poisons and poisoned weapons (Smart, 1997). The British use of picric acid–filled shells during the Boer War and the Japanese use of arsenical rag torches in the Russo-Japanese War further illustrate that chemical warfare was considered by some a legitimate form of warfare at the turn of the 20th century (Smart, 1997). During the early 20th century, technological advancements in the chemical industry made the possibility of sustained military operations using chemicals a realistic possibility. The murder of Archduke Franz Ferdinand in Sarajevo in June 1914, which sparked World War I, set the stage for what would become the first widespread use of chemical weapons to date (Harris and Paxman, 2002).

2.2 The first sustained use of chemicals as agents of war

The talk and rhetoric of the late 19th century should have prepared the countries on both sides of World War I for chemical warfare. However, that was not the case (Smart, 1997). World War I clearly demonstrated the deadly and destructive nature of chemicals in modern warfare. Both sides of the war experimented with novel forms of warfare, including chemical weapons, and followed the lead of their adversary (Hay, 2000). It is little wonder that this war is known as the "chemist's war" (Fitzgerald, 2008). Initially, the French used gas grenades with little effect, followed by the German use of shells filled with tear gas (Joy, 1997). The Germans, capitalizing on their robust chemical industry, produced shells filled with dianisidine chlorosulfate (Smart, 1997). These shells were used in October 1914 against the British at Neuve-Chapelle, but with little effect. In the winter of 1914–15, the Germans fired 150-mm howitzer shells filled with xylyl bromide (Smart, 1997). These shells were fired on both the eastern and western fronts, with disappointing effects. Despite this inauspicious start, efforts were continued to develop new uses of chemical warfare. It would soon be evident that chemical weapons would be devastating on the battlefield (Coleman, 2005; Tucker, 2006). In late 1914, Fritz Haber, a German scientist who later won the Nobel Prize in Chemistry, proposed the possibility of releasing chlorine gas from cylinders (Joy, 1997). Chemical warfare was attractive to Germans for two reasons: the shortage of German artillery shells and the ability to defeat the trench system of the enemy (Smart, 1997). After consideration and debate, the Germans released chlorine in April 1915 at Ypres, Belgium (Coleman, 2005). The German military was not prepared for the tremendous operational advantage the chlorine release provided, however. It did not take long for the British and French forces to respond in kind to the German offensive (Vedder and Walton, 1925; Joy, 1997; Smart, 1997; Coleman, 2005). In the fall of 1915, a British officer, William Livens, introduced a modified mortar (Fig. 2.1) that could project gas-filled shells of chlorine or phosgene, the two agents of choice at that time (Joy, 1997). Both chlorine and phosgene caused extreme respiratory problems to those soldiers who were exposed to them (Vedder and Walton, 1925; Joy, 1997; Smart, 1997; Coleman, 2005; Hurst et al., 2007) (Fig. 2.2).

FIGURE 2.1 British Livens Projector, western front, World War I.

As the United States entered the war in the spring of 1917, an obvious concern of its military command was the effect of chemical warfare on standard operations. Chemistry departments at universities were tasked with investigating and developing novel chemical agents (Joy, 1997).

Protective equipment (Fig. 2.3) and basic studies of the biological effects of chemical agents were assigned to the US Army Medical Department (Joy, 1997). In the fall of 1917, the army began to build an industrial base for producing chemical agents at Edgewood Arsenal, MD (Joy, 1997). As the effects of chlorine and phosgene became diminished by the advent of gas masks (Fig. 2.4), the Germans turned to dichlorethyl sulfide (sulfur mustard) at Ypres against the British (Joy, 1997).

FIGURE 2.2 Australian infantry in trench with gas masks donned, Ypres, Belgium, September 1917.

FIGURE 2.3 US Army captain wearing a gas mask in training, 1917.

FIGURE 2.4 World War I soldier and horse, both wearing gas masks.

As opposed to the gases, sulfur mustard remained persistent in the area, and contact avoidance was the major concern (Joy, 1997). It is worth noting that almost 100 years after it was first used on the battlefield, sulfur mustard still has no effective treatment; research continues into developing effective therapeutics (Babin and Ricketts, 2000; Baskin and Prabhaharan, 2000; Casillas et al., 2000; Hay, 2000; Schlager and Hart, 2000; Hurst et al., 2007; Romano et al., 2008). It has been estimated that there were over 1 million chemical casualties (Fig. 2.5) of World War I, with almost 8% being fatal (Joy, 1997). The Russians on the eastern front had a higher percentage of fatalities than other countries in the war, primarily due to the later introduction of a protective mask (Joy, 1997). The relatively low rate of chemical deaths in World War I demonstrated the most insidious aspect of the use of such weapons—namely, the medical and logistical burden that it placed on the affected army. The eventual Allied victory brought a temporary end to chemical warfare. In 1919 the Treaty of Versailles prohibited the Germans from producing or using chemical weapons.

2.3 Initial countermeasures

The concept of a protective mask against chemical attack dates back over 500 years, to Leonardo da Vinci (Smart, 1997). By the mid-19th century, protective masks were proposed in the United States and Europe for both industrial and military use. The modern gas mask was developed by the Germans with sodium thiosulfate- and

FIGURE 2.5 British soldiers temporarily blinded by tear gas awaiting treatment at the Battle of Estaires, April 1918.

bicarbonate-soaked pads, and it was used during World War I (Joy, 1997). The French and English soon followed with their own versions of gas masks (Joy, 1997). In 1916 the Germans introduced a mask that incorporated a canister through which the soldiers breathed (Joy, 1997). Initially, the American forces in World War I used gas masks obtained from allies already fighting in the war (Smart, 1997). In 1918 the Americans introduced Richardson, Flory, and Kops (RFK) mask, a modified version of the British mask. In addition, masks were developed for the animals, such as horses, that supported the war efforts. Decontamination efforts during World War I were rudimentary and included chemical neutralization and aeration of clothing and equipment. Although the need to detect chemical agents was clearly identified, very little progress was made during World War I. Medical treatment included removal of the patient from the source, decontamination, and palliative care (Smart, 1997).

2.4 Events after World War I

By the time World War I ended, the world had been introduced to chemical warfare on an unprecedented level. While some groups thought that humanity had learned a lesson about the cruel nature of chemical warfare, others prudently went to work on improving chemical defenses (Vedder and Walton, 1925). The thoughts of many professional military officers were that future wars would be fought under the new paradigm of chemical warfare (Vedder and Walton, 1925; Vedder, 1926; Smart, 1997). New gas masks were developed, and training in chemical environments was introduced (Vedder and Walton, 1925; Vedder, 1926; Joy, 1997). Textbooks and manuals, such as those written by US Army Colonel Edward B. Vedder (Fig. 2.6), were introduced into the military medical community (Vedder and Walton, 1925). In addition, the civilian medical community gained valuable insight into toxicology from the events of World War I (Vedder, 1929; Johnson, 2007). Despite the firsthand experience with chemical warfare, some countries, including the United States, struggled to fund their offensive and defensive programs adequately during demobilization (Smart, 1997).

It did not take long for chemical warfare to appear in other conflicts. Chemical agents were used to subdue rioters and suppress rebellions. For example, the British used chemical agents to suppress uprisings in Mesopotamia in the early 1920s by dropping bombs in cities throughout the area (Coleman, 2005). The Soviet Union used chemical agents to quell the Tambov peasant rebellion in 1921, and France and Spain used sulfur mustard-gas bombs to subdue the Berber rebellion in the 1920s (Werth et al., 1999). Italy and Japan used sulfur mustard in several small regional conflicts (Joy, 1997). The Italian conflict in Ethiopia was particularly noteworthy because sulfur mustard was sprayed and dropped from planes, and some experts think that the agent's use contributed significantly to the Italian victory (Smart, 1997). This use demonstrated the contemporary belief that chemicals were viable alternatives to traditional combat.

The Japanese also used chemical weapons during the 1930s against regional foes. Sulfur mustard gas and the vesicant lewisite were released against Chinese troops and were also used in Southeast Asia (Coleman, 2005). Lewisite is an arsine that was usually produced as an oily brown liquid that was said to smell like geraniums (Spiers, 1986; Hammond, 1994). It was developed in the United

FIGURE 2.6 Captain Edward B. Vedder, the "father" of the United States Army Medical Research Institute of Chemical Defense (USAMRICD).

States by Winford Lee Lewis in 1918 and was found to be effective at penetrating clothing. The United States produced approximately 20,000 tons of lewisite but only used small quantities of the chemical in World War I (Coleman, 2005). Dimercaprol, more commonly called British antilewisite, was developed as an effective treatment for the vesicant (Goebel, 2008). In the period between the two world wars, sulfur mustard was a key part of defensive planning (Coleman, 2005). New stores of sulfur mustard were produced in many countries. Work continued on many fronts to improve protective equipment. For example, the US Chemical Warfare Service introduced the M1A2 mask, an improvement on the M1 mask (Smart, 1997). In the Geneva Protocol of 1925, 16 of the world's major nations pledged never to use gas as a weapon of warfare; however, it was not ratified in the United States until 50 years later, in 1975 (Hammond, 1994). There has long been vigorous debate on the merits of treaties with nations that balance military needs against the potential irrational concept of chemical warfare (Vedder, 1926).

2.5 World War II

In the lead-up to World War II, the Germans forever changed chemical warfare with the discovery of the organophosphorus nerve agents (Goebel, 2008). These agents inhibit cholinesterase enzymes in the nerve synapse responsible for the breakdown of the neurotransmitter acetylcholine (ATSDR, 2019). This results in the accumulation of the neurotransmitter in the synapse and overstimulation of the nervous system. This can result in subsequent respiratory failure and death (ATSDR, 2019).

In 1936 Gerhard Schrader, a German chemist working on the development of insecticides for IG Farben, developed a highly toxic organophosphate compound, which he named tabun (Hersh, 1968; Hammond, 1994). Schrader and an assistant became casualties of their discovery when a drop of the neurotoxicant was spilled in the lab, exposing both of them (Tucker, 2006). Had the amount of tabun spilled been greater, both researchers would have certainly succumbed to the effects of the poison. Tabun was the first of a series of compounds termed nerve gases (Coleman, 2005). The correct terminology, however, is nerve agents, as these substances are not gases; rather, they are liquids dispersed as fine aerosols. Tabun was extremely toxic in small amounts, and it was invisible and virtually odorless (Tucker, 2006). The compound could be inhaled or absorbed through the skin. These characteristics made it too dangerous to be used as an insecticide by farmers. German law required that any discovery having potential military applications be reported to military officials (Tucker, 2006). Schrader was not overly excited about producing chemical agents for the military; however, the Germans placed him in a secret military research facility with the emphasis on producing these nerve agents and discovering new agents (Tucker, 2006). Subsequently, Schrader and his team of researchers discovered a more lethal organophosphate compound similar to tabun, which he named sarin in honor of the team members: Schrader, Ambrose, Rudriger, and van der Linde (Coleman, 2005).

At the onset of World War II, both the Allies and the Germans anticipated that chemical agents would be deployed on the battlefield (Tucker, 2006). This expectation intensified research into the development of new agents, delivery systems, and methods of protection (Figs. 2.7 and 2.8). The Allied forces were unaware of the Germans' new nerve agent, tabun, at the beginning of the war. The German Army advanced very rapidly across Europe using their Blitzkrieg method of maneuvering. As a result, German military leaders were reluctant to use chemical weapons, fearing that their forces would lose momentum waiting for contaminated areas to clear. (Tucker, 2006). Nevertheless, the Germans produced and stockpiled large amounts of nerve agents throughout the war (Spiers, 1986). The production of these organophosphate agents was complex, required custom equipment, and was hazardous to those involved in its production (Tucker, 2006). If workers got exposed, they would be dunked in a bath of sodium bicarbonate (Harris and Paxman, 2002; Goebel, 2008). It is also interesting to

FIGURE 2.7 Gas mask production—Detroit, Michigan, 1942.

FIGURE 2.8 A private trains using protective gear during World War II.

note that some members of the German workforce were given rations containing higher percentages of fat (Harris and Paxman, 2002). This was done because authorities observed that workers with higher quality rations seemed protected against exposure to low levels of tabun. Many detainees were used in the manufacture and testing of chemical agents in Germany (Harris and Paxman, 2002; Tucker, 2006). It is not known how many chemical casualties resulted from prisoners of war being forced to work at producing nerve agents, but some fatalities were documented. The discovery of tabun and sarin was followed by the discovery of soman in 1944 by Richard Kuhn and Konrad Henkel at the Kaiser Wilhelm Institute for Medical Research (Tucker, 2006). This class of nerve agents is collectively termed "G" agents; the G stands for German, since German researchers discovered this class of compounds. A second letter is included as the specific identifier of each compound: GA (tabun), GB (sarin), GD (soman), and GF (cyclosarin) (ATSDR, 2019). These agents were mass-produced by the Nazi regime throughout the war, but they were not used (Tucker, 2006). There has been considerable debate about why the Germans did not employ their chemical weapons in World War II. While it may never be known conclusively, several possible reasons include lack of intelligence regarding the German superiority in chemical weapons, fear of retaliation, and Adolf Hitler's personal exposure to chemical agents on the battlefield in World War I (Harris and Paxman, 2002; Tucker, 2006). Other chemical agents that had been produced during and following World War I were still being produced. On December 2, 1943, German planes sank several American ships off the coast of Italy. At least one of the ships contained sulfur mustard, which was to be used in retaliation if the Germans unleashed a large-scale chemical weapons attack (Tucker, 2006). Many casualties resulted from exposure to the sulfur mustard, some of which included civilian merchant seamen (US Navy, 2019). The presence of the agent on the ship was classified, resulting in physicians incorrectly treating many of the victims (Tucker, 2006).

2.6 Post–World War II

By the conclusion of World War II, both the Allies and Germany had stockpiled large amounts of chemical agents (Tucker, 2006). The Allied forces divided up the stockpiles of agents discovered in German facilities. Following the end of the war, many of the Allied countries continued to conduct research on the German nerve agents. The rise of the Soviet Union as a power and adversary prompted the United States and other countries to continually search for novel chemical and biological warfare agents (Tucker, 2006). The research and resources that were allotted for these efforts were not trivial, even though they were often overshadowed by the research and development of thermonuclear weapons (Hersh, 1968; Goebel, 2008).

The post–World War II era ushered in the nuclear age. Some felt the age of chemical warfare was over (Smart, 1997), but subsequent events would prove this to be a hasty conclusion. In the United States, research on the G-series agents and medical countermeasures against these agents was accomplished by the late 1940s. Research and intelligence gathering were further hastened by the impressive gains that the Soviet Union made in

chemical warfare capability in the years after World War II. By the early 1950s, production of sarin had been initiated in the United States (Smart, 1997). At nearly the same time, Ranajit Ghosh, a chemist at the British company Imperial Chemical Industries plant, developed a new organophosphate compound to use as a potential insecticide (Tucker, 2006). Like with Gerhard Schrader, this compound was deemed too toxic to be used in the field as a pesticide. The compound was sent to researchers in Porton Down, England, synthesized, and developed into the first of a new class of nerve agents, the V agents (Goebel, 2008). Like the G agents, the V agents have a second letter designation: VE, VG, VM, and VX (Coleman, 2005). Of these, VX was the most common. The V series of agents are generally more toxic than the G agents (ATSDR, 2019). In a deal brokered between the British and US governments, the British traded the VX technology for the thermonuclear weapons technology of the United States (Tucker, 2006). The United States produced and stockpiled large quantities of VX after that (Hersh, 1968; Hammond, 1994).

Throughout the 1950s and 1960s, advancements were made in the production and delivery of chemical weapons to include sarin and VX (Smart, 1997). While work on improving masks continued, a renewed concern was the inability to detect nerve agents. Several prototypes were developed in the mid-1950s. Great advancements were made in the therapeutics of agents that inhibited the enzyme acetylcholinesterase (Taylor, 2006; Gupta, 2008; Klaassen, 2008). Atropine was introduced in the early 1950s. Oximes were added as an adjunct to speed up reactivation of the enzyme (Smart, 1997). The autoinjector was developed to overcome user fear of self-injection of atropine. Major advances were made in the use of chemical weapons in artillery (Fig. 2.9). For example, the United States developed both short- and long-range rockets filled with chemical agents. But it disposed of stockpiles of its chemical weapons in the late 1960s in an operation termed CHASE (which stood for "cut holes and sink 'em") in the sea (Coleman, 2005). In 1969 nerve agent stockpiles were discovered in US depots in Japan after several US military members became ill while doing maintenance (Tucker, 2006). This stockpile, which had been kept secret from the Japanese, created an uproar that later resulted in the disposal of the agents in the Johnston Atoll in the Pacific Ocean.

Defensive equipment, such as improved field alarms and drinking tubes for gas masks, were introduced in the 1960s (Smart, 1997). Great strides were also made in collective protection during the 1960s and 1970s. Although not used extensively since World War I, chemical agents have nonetheless been used for military purposes. The Egyptians allegedly used sulfur mustard and possibly nerve agents in the North Yemen Civil War in the 1960s

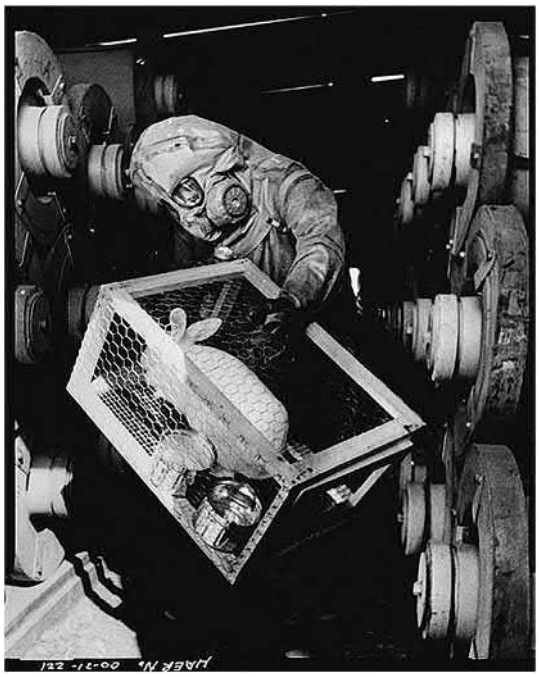

FIGURE 2.9 Testing for leaks at a sarin production plant, 1970.

(Joy, 1997; Smart, 1997). This was the first reported use of nerve agents in armed conflict. There were allegations that chemical agents were used by the Vietnamese in Laos and Kampuchea in the late 1970s (Coleman, 2005). In the Vietnam War, the United States used defoliants and tear gas, and the Soviet Union was accused of using chemical agents in their war in Afghanistan (Joy, 1997).

2.7 Incapacitants and toxins

Incapacitating agents have long been considered an intermediate between chemical and traditional warfare. The Germans investigated the military use of lacrimators in the 1880s followed shortly thereafter by the French (Smart, 1997). The English and French considered using lacrimators in World War I (Smart, 1997). Japanese forces used tear gas against the Chinese in the late 1930s. The US Army used riot control agents and defoliants in the Vietnam War (Smart, 1997). The defoliant known as Agent Orange was later potentially linked to several forms of cancer (Stone, 2007). During the 1950s and 1960s, the United States had an active incapacitant program (Smart, 1997). These agents were thought of as more humane than traditional chemical agents because the intent was not to kill. These agents were designated K agents and included tetrahydrocannabinol and lysergic acid (Smart, 1997). One of the most extensively studied incapacitating agents was 3-quinuclidinyl benzilate, designated BZ by the US Army (Ketchum, 2006). Like many incapacitating agents, BZ was not adopted due to

difficulty producing reproducible effects, unwanted side effects, latency in its effects, and difficulty in producing a dissemination that was free of smoke (Smart, 1997; Ketchum, 2006).

There have been multiple attempts to use the toxins from plants and living organisms to develop viable weapon systems. Two that are noteworthy are ricin and botulinum toxin. Ricin, a very potent toxin derived from the castor bean plant, has been recognized as a potential biological weapon since World War I. While the British were developing the V agents, US military researchers patented a procedure for purifying ricin (Harris and Paxman, 2002). The development of a method of disseminating ricin as a chemical weapon proved problematic, which made its use very limited. In 2003 ricin was detected on an envelope processed in a postal facility in Greenville, South Carolina. Postal workers did not develop symptoms of ricin exposure, and the individual who mailed the letter remains at large (Shea, 2004). The development and use of botulinum neurotoxin as a biological weapon was initiated at least 60 years ago (Smart, 1997; Arnon et al., 2001). In the 1930s, during Japan's occupation of Manchuria, the Japanese biological warfare group Unit 731 purportedly fed cultures of *Clostridium botulinum* to prisoners, killing them. The US Army biological weapons program produced botulinum neurotoxin during World War II in response to Germany's biological weapons program (Coleman, 2005). In fact, more than 100 million toxoid vaccine doses were prepared in time for the D-Day invasion of Normandy (Arnon et al., 2001).

2.8 Recent experience

The 1980s proved to be a very significant time for the employment of chemical weapons on the battlefield. In 1980 Iraq invaded Iran (Smart, 1997). The Iraqi armed forces, advised by the Soviet Union, possessed chemical agents and were trained in their use. The war was unequivocally barbarous, and neither side gained an advantage. In many ways, this war had similarities to World War I. By 1983, Iran formally protested to the United Nations (UN) about the Iraqi use of chemical agents. The general consensus was that Iraq used sulfur mustard agents and possibly tabun in this war (Fig. 2.10). It is estimated that 5% of Iranian casualties, totaling approximately 45,000, can be attributed to chemical agents; the Iraqi Army used chemical agents against the Kurdish minority in northern Iraq as well; and Libya was suspected of using chemical agents when it invaded Chad in 1986 (Smart, 1997).

The late 1980s also saw improvements in defensive equipment, such as the M40 gas mask developed by the United States (Smart, 1997). Other advancements were made in collective protection, decontamination, and detection. In 1984 US President Ronald Reagan issued a statement calling for an international ban on chemical weapons (Tucker, 2006). Subsequently, on June 1, 1990, President George H.W. Bush and Soviet leader Mikhail Gorbachev signed a treaty banning the production of chemical weapons and initiated the destruction of the stockpiles of both nations (Tucker, 2006). In 1993 the Chemical Weapons Convention was convened and signed, and it was implemented in 1997 (Hammond, 1994). As of 2008, the vast majority of UN member states had joined the Chemical Weapons Convention (OPCW, 2008).

In 1990 the Iraqi Army invaded neighboring Kuwait. Subsequently, the United States, at the request of Saudi Arabia, led a coalition to send forces to the area (Smart, 1997). These forces were the largest to operate in a potential chemical environment since World War I. They were provided with atropine autoinjectors, an acetylcholinesterase reactivator, and a nerve agent pretreatment

FIGURE 2.10 Aftermath of Iraqi chemical weapon attack (1980s).

(pyridostigmine bromide). Fortunately, chemical weapons apparently were not used in this conflict, although multiple false alarms were reported. The failure of the Iraqi military to use chemical weapons could be attributed to fear of retaliation, breakdown of communication, changing wind patterns, the surprising speed of the coalition attack, or the fact that Iraqi chemical infrastructure was attacked during the initial portion of the conflict. Since the conflict ended, many coalition veterans have reported a myriad of symptoms that have been commonly referred to as Gulf War syndrome. The etiology of this syndrome is unclear despite multiple epidemiological studies (Coleman, 2005). The most recent example of chemical weapons use is the ongoing Syrian Civil War (Pellerin, 2013). Both sarin and sulfur mustard have been reportedly been used in this conflict (Sezigen, 2019; Kilic et al., 2018).

2.9 Terrorist use

One of the reasons why chemical weapons have been used relatively infrequently in combat over the past century is the fear of retaliation by opposing countries. In less organized asymmetrical conflicts, this fear is not as dangerous. At the same time, the potential exploitation of chemical weapons by terrorists is of great worldwide concern (Kilic et al., 2018). The appeal of these weapons to terrorists lies largely in the fact that many of these chemical agents are cheap and relatively easy to produce, transport, and release. These characteristics, along with the fear associated with the idea of a chemical attack, make chemicals an ideal weapon for terror attacks (Romano and King, 2001). In 1974 Muharem Kurbegovic attacked several public buildings with firebombs in California and claimed to have developed sarin and some other nerve agents (Tucker, 2006). The search of his home resulted in the discovery of various precursor materials for chemical agents and a large amount of sodium cyanide. In 1994 the Aum Shinrikyo, a Japanese religious cult, carried out several attacks both in the subway and in residential areas using sarin produced by the cult's members (Tucker, 2006). A total of 19 people were killed, and over 6000 received medical attention, some of which suffered long-term neurological and behavioral deficits (Yokoyama et al., 1998). Some of those who sought medical attention may have done so due to a fear of exposure. Psychological stress is a common aftermath of a chemical or biological attack (Romano and King, 2001). In the 21st century, chemicals that once had been used exclusively by the military have reemerged as contemporary threats. In the fall of 2006, Al Qaeda and associated groups used chlorine combined with traditional car and truck bombings to spread panic in Iraq (Garamone, 2007). These attacks were followed by similar incidents in the subsequent months. There have also been reports of state-sponsored terrorist activities to eliminate individuals using chemical weapons as agents (Hersman and Pittinos, 2018) such as the use of the nerve agent, VX, to assassinate Kim Jong Nam, the brother of North Korean leader Kim Jong Un, at Kuala Lumpur international airport in 2017 (Bradley, 2017) and the use of a Novichok agent (an extremely toxic organophosphate nerve agent) in the poisoning of former Russian double agent Sergei Skripal and his daughter Yulia in England in 2018 (Peplow, 2018).

2.10 Concluding remarks and future directions

So long as there are legitimate uses for chemicals in our society, the risk of chemical agents in conflict and terrorist activity will always be present. Research continues across the globe for better detection, protection, and treatment of chemical agents. While many countries have denounced and are signatories to various treaties to limit the use and production of chemical warfare agents, nonstate and terror organizations are under no such restrictions. Luckily, chemical weapon use has been limited in both warfare and less formal conflicts. As we progress into the 21rst century, the use of established chemical warfare agents is a real possibility. The potential use of legitimate industrial chemicals (e.g., the Iraqi burning of petroleum fields in the first Gulf War) and the potential synthesis of new agents should also be recognized (Kilic et al., 2018). History has demonstrated that chemicals have been used in both organized and asymmetrical conflicts, and preparations for defense and therapy for such encounters is prudent. Despite the progress made during the past 100 years, chemicals represent a unique force multiplier that simply cannot be ignored in the 21st century.

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Chapter 3

Global impact of chemical warfare agents used before and after 1945

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3.1 Introduction

The threat of chemical weapons (CWs), used either by states or parties to the Chemical Weapons Convention (CWC; Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and on their Destruction) or by terrorists, has never attracted so much public attention as it has in the past 10 years. Despite the existing legal documents dealing with the prohibition of CWs, for example, Geneva Protocol 1925 and CWC, some incidents of the use of CWs in different conflicts and terrorist attacks have been observed. Moreover, the alleged use of CWs has been noted during the period from 1925 to the present. It must be emphasized that the theoretical and practical basis for production, storage, and use of CWs still exists. Also, it must be clearly stated that CWs are applicable at any time, in any place, and in large quantities.

CWs consist of chemical warfare agents (CWAs) and the means them to deliver to the target. They are characterized by high effectiveness for use against large targets and are known as area weapons or silent weapons. They are relatively low cost and it is possible to achieve destruction of everything that is living while avoiding the destruction of materials and buildings. They are also called the nuclear weapons of poor countries—a "poor man's nuclear weapon." It should be pointed out that the use of CWs is connected with the use or release of toxic chemicals; thus chemical warfare can be considered part of generally observed situations in which toxic chemicals are used or released and influence the environment and humankind.

A number of causal reasons for these events exist but, apart from accidents connected with the release of toxic chemicals from a natural source (e.g., volcanoes), the factors shown in Fig. 3.1 or their combinations can be involved.

For military purposes a number of chemicals were tested, but only a few are contained in military arsenals. However, according to the definition used by the CWC, any toxic chemical intended for military use must be considered a CW; in other words, the aim is to limit the designation of the compound in question for use as a CW. However, it is possible for terrorists to choose any chemicals with high toxicity.

3.2 Background

The use of toxic chemicals against humankind is as old as warfare. The use of the poisoned arrow against humans—not animals—can be considered as the beginning of chemical warfare and is characterized as the intentional use of chemicals.

At the very beginning, chemical warfare was more closely connected with fire. "Greek fire" was an excellent naval weapon because it would float on water and set fire to wooden ships. There are other examples from history; for example, toxic smoke was used in China in 2000 BCE. In Thucydides' *History of the Peloponnesian War* (the 5th century BCE war between Athens and Sparta), we find the first description of chemical warfare—the formation of toxic sulfur oxide by burning sulfur. In the year 184 BCE, Hannibal of Carthage used baskets with poisonous snakes against his enemy. Both Socrates and Hamlet's father were poisoned with coniine. Aqua Toffana containing arsenic was also a known poison in ancient Italy. Leonardo da Vinci proposed a powder of arsenic sulfide in the 15th century. There are many more

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examples of the use of CWAs (Bajgar et al., 2007b). Modern history shows us that terrorists have used other chemicals, such as ricin (the Bulgarian, G. Markov, was poisoned in 1978) or dioxin (the President of Ukraine, Viktor Andriyovych Yushchenko, was poisoned in 2004).

In a region of Bohemia, a "form" of CW was used as early as 600 years ago. It was in 1422 that the castle of Karlstein, the property of King Charles IV, was besieged and 1822 kegs containing matter from the cesspools of the streets of Prague were hurled into the castle. Allegedly, the stench in the castle was unbearable. According to historical sources, the castle defenders were probably intoxicated with hydrogen sulfide released from the contents of the cesspools and therefore they showed typical symptoms of poisoning (Bajgar, 2006).

There were some attempts to prohibit CWs by international agreement or law. Most early attempts were bilateral or unilateral agreements directed at the use of poisons. These included the 1675 agreement between France and Germany, signed in Strasbourg, to ban the use of poison bullets.

The first international attempt to control chemical and biological weapons took place in Brussels in 1874, when the International Declaration was signed and included a prohibition against poison and poisoned arms. Despite the first and second Brussels and Hague Conventions (1899 and 1907 signatories agreed not to use projectiles that could spread asphyxiating or deleterious gases), the world witnessed the application of chemicals during warfare to an unprecedented extent during World War I (WWI). A brief summarization of the events connected with the use/release of toxic chemicals is given in Table 3.1.

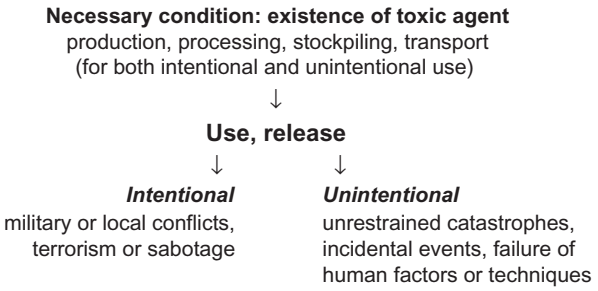

FIGURE 3.1 Possible reasons for release/use of toxic chemicals.

TABLE 3.1 Some milestones related to the use/release of CWs and toxic chemicals.

Year(s)	Event
2000 BCE	Toxic smoke in China inducing sleep
4th century BCE	Spartacus—toxic smoke
184 BCE	Hannibal—baskets with venomous snakes
1168	Fustat (Cairo)—use of "Greek fire"
1422	Bohemia region—cesspools (H_2S)
1456	Belgrade—rats with arsenic
19th century	Admiral Dundonald—proposed the use of chemicals in war
1914–18	WWI—start of chemical war
1918–39	Development of new CWs and protective means
June 17, 1925	Geneva Protocol
December 23, 1936	Lange and Kruger—synthesis of tabun
1940–45	Concentration camps—cyanide
1943	Synthesis of sarin
1943	Hoffmann and Stoll—synthesis of LSD-25
1945	Kuhn—synthesis of soman
1950	V agents are invented
1961–68	Production of VX
1961–71	Vietnam War—herbicides (impurity dioxin)

(*Continued*)

TABLE 3.1 (Continued)

Year(s)	Event
1962	BZ was introduced into military arsenals
1970	Bicyclic phosphates considered as potential CWAs
1976	Seveso—release of dioxin
1980	Some rumors on intermediate volatility agent
1984	Bhopal incident—release of methylisocyanate
1985	Decision on production of binary CWs
1986, 1987	Demonstration of US CWs (Tooele) and Soviet Union CWs (Shikhany) to the CD in Geneva
1987	Production of binary CWs
1988	Halabja—use of mustard
1980–90	Rumors of new nerve agent Novichok
1989	Conference on chemical disarmament, Paris
1991	Persian Gulf War—veteran's syndrome
1992	BZ military stocks of the United States were destroyed
1992	Finalization of the rolling text of the CWC at the CD—Geneva
1993	Signing CWC in Paris
1993	Preparatory Commission on OPCW
1994	CWs of Iraq were destroyed
1994	Aum Shinrikyo—sarin attack in Matsumoto
1995	Aum Shinrikyo—sarin attack in Tokyo
April 29, 1997	CWC—entry into force; establishment of OPCW in The Hague
2000	Research on nonlethal weapons intensified
2002	Moscow theater—fentanyl derivatives used against terrorists
April 29, 2012	CWs of the state parties to the CWC to be destroyed but it was prolonged; this period varies from 2015 to 2023 years
August 2013	Syria—use of sarin
February 2017	Kim-Chang Nam assassination by V-type agent at Kuala Lumpur airport
March 2018	Skripal and his daughter, percutaneous intoxication by Novichok, Salisbury
September 2018	Destruction of Russian CWs completed

3.3 Military use of chemical weapons

The intentional use of CWs for military purposes can be found in both global and local conflicts. A typical example is the warning "Gas! Gas!" This was common in WWI and it is well known from the E.M. Remarque novel *All Quiet on the Western Front* in which Remarque describes a chemical attack with chlorine.

During WWI, many chemicals were used, including mustard and asphyxiating and irritant agents. Approximately 45 types (27 more or less irritating and 18 lethal) of toxic chemicals were used. During the latter part of 1914, irritants were used by Germany and France; the effect was insubstantial. In late 1914, Nobel Prize winner Fritz Haber of the Kaiser Wilhelm Physical Institute in Berlin (chemical synthesis of ammonium in 1918) came up with the idea of creating chlorine, although this idea of using toxic chemicals in war was expressed by Admiral Dundonald as early as 1855. Chemical warfare really began in 1915, when German troops launched the first large-scale poison gas attack at Ypres, Belgium, on April 22, using 6000 cylinders to release 168 tons of chlorine gas, killing 5000 British, French, and Canadian soldiers. This date is recognized as

"the birthday of modern chemical warfare," and thereafter the belligerent parties frequently used chemical gases against each other. Phosgene was introduced by Germany in late 1915. Soon after the first chlorine attack, the Allies used primitive emergency protective masks. In May 1916, the Germans started using diphosgene, while the French tried hydrogen cyanide 2 months later and cyanogen chloride the same year. The first time mustard gas was used by German troops was July 12, 1917. After its use near Ypres, it was also called yperite.

By the end of WWI, approximately 124,200 tons of CWAs (chlorine, phosgene, mustard, etc.) had been released, causing at least 1.3 million casualties, of which more than 90,000 were fatal. The threat of the use of CWAs led to the development of protective means not only for humans but also for horses and dogs. The effectiveness of CWs in comparison with classic munition was evident: 1 ton of classic explosives caused 4.9 casualties; 1 ton of chemical munitions caused 11.5 casualties; and 1 ton of yperite caused 36.4 casualties (Bajgar, 2006).

3.4 The period between World War I and World War II

The terrible casualties from the CWs used during WWI and the dangerous consequences for humans and the environment led to the signing of the "Geneva Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous and other Gases and Bacteriological Methods of Warfare" on June 17, 1925. This is recognized as one of the unique and famous international treaties on the prohibition of CWs. However, it neither comprises provisions for effective verification nor prohibits the development, stockpiling, and transfer of CWs. Moreover, no definition of CWs was included. Despite the provisions of the Geneva Protocol, during 1935–36 Italian troops used CWs during their invasion of Abyssinia (Ethiopia). This first major use of CWs after WWI came after October 3, 1935, when Mussolini launched an invasion of that country. Despite the Geneva Protocol (Italy had ratified in 1928), the Italians used mustard gas with horrible effects. Later, CWs were used between Japan and China during 1937–45. The Japanese attacked Chinese troops with mustard gas and lewisite. The Japanese, in addition to their biological program, had an extensive CWs program and were producing agents and munitions in large quantities by the late 1930s.

3.5 World War II

Despite the storing and stockpiling of CWs by the great powers engaged in World War II (WWII), these fatal weapons were not significantly used (except small examples) during WWII (probably because of the fear of massive retaliatory use of CWs). An example of intentional use, but not during military conflict, was the killing of prisoners in concentration camps in Nazi Germany. The agent first used in the camps was carbon monoxide, followed by the more "effective" hydrogen cyanide released from Zyklon B. Some experiments with aconitine-impregnated shells and some other toxic compounds including biological agents were tested on prisoners.

However, during WWII, an important step in the preparation of the most dangerous CWA was observed in Germany. In Schrader's group, organophosphates (Ops) were synthesized, primarily with the aim of obtaining more effective insecticides. Between 1934 and 1944, Schrader's team synthesized approximately 2000 Ops, including two well-known OP compounds, parathion and paraoxon. As early as 1935, the government of Nazi Germany insisted that Schrader switch the primary aim from OP insecticides to CWAs. Presently, Ops are widely used in agriculture, medicine (human and veterinary), and industry. These compounds also include nerve agents (the most toxic compounds of the OP group). Nerve agents such as sarin, tabun, soman, and VX are the main compounds of CWAs. The Germans were also the greatest producers of nitrogen mustard and produced approximately 2000 tons of HN-3.

This part of history is well known (Koelle, 1963, 1981; Bajgar, 2006; Tuorinsky and Lenhart, 2008; Klement et al., 2013). The first synthesis of OP was described in the second half of the 18th century. For a long time the first OP (its toxicity was described later) was considered to be TEPP, which was synthesized by Clermont (1854–55). Philippe de Clermont was a well-known chemist in Sorbonna. Charles Adolph Wurtz dedicated his work to the synthesis of esters of pyrophosphoric acid. These data were specified by Petroianu (2008), and thus he contributed to the discovery that the first synthesis of this OP—TEPP—was performed by Vladimir Moshnin of Moscau. These data are depicted in the work of Patočka (2010). New trends in the synthesis of nerve agents have been described by Halamek and Kobliha (2011).

Tabun was synthesized in 1936, followed by others (sarin at the end of WWII, followed by soman), and production of these agents for the military in large quantities and their stockpiling were recognized after WWII in Dyhernfurth, Poland (e.g., stocks of tabun and some quantities of sarin). The technology was subsequently transferred to Russia, and research and development of new OP nerve agents was continued. During this period, British and American scientists were evaluating the toxic properties of DFP.

3.6 The period after World War II, and the Cold War

At the end of WWII, many Allied nations seized the German CWs. Most of the CW manufacturing plants in

Germany were taken over and moved to new sites in Russia, such as the military area of Shikhany. This "take-over" prompted other states to begin even more research into CWs. Despite the Allies' own research into CWs, very important technologies and "know-how" were obtained from Nazi Germany for both the United States and the former Soviet Union.

The interest in CW technology was probably one reason for the change to the future border: according to Churchill's history of WWII, the proposed future boundary between Poland and Germany had been primarily agreed to consist, in part, of the Oder River flowing to the Baltic Sea, and its tributary, the Neisse River. Before their confluence, the Neisse consisted of two branches, the East Neisse and the West Neisse. The East Neisse should be the boundary, resulting in slightly more territory for Germany. Stalin held for the West Neisse and progress was delayed. No one knows why Stalin was so insistent in this matter. The reason was probably very simple: the small town of Dyhernfurth (now Brzeg Dolny), a few kilometers north of Breslau (Wroclaw) in the disputed territory, contained a factory for the production of nerve agents. It was estimated that when Dyhernfurth was captured it contained stockpiles of 12,000 tons of tabun, 600 tons of sarin, and an unknown amount of soman. Presumably, the factory was dismantled and, along with their stockpiles, transported to the Soviet Union (Koelle, 1981). It has been documented that the Soviets were ready to conduct a chemical attack and their research and development of CWs were intensified.

In the United States, during the 1950s, the chemical corporations concentrated on the weaponization of sarin. At the same time, they became interested in developing CWs that incapacitated rather than killed the targets. Mescaline and its derivatives were studied but without practical output. Five years later, the new project "Psychochemical Agents" (later K-agents) was established. The objective was to develop a nonlethal but potent incapacitant. Nonmilitary drugs like LSD-25 and tetrahydrocannabinol were also examined. None of these agents was found to be of military importance. The first and only incapacitant was BZ, developed in 1962; however, its stocks were destroyed in 1992, as declared by the US delegation to the Conference on Disarmament in Geneva (Document of CD, 1991). These agents, intended not to kill but to induce incapacity, are covered under the class of nonlethal weapons (Hess et al., 2005).

In the former Soviet Union as a whole, during 1940–45, approximately 110,000 tons of first-generation toxic chemicals were produced, and most of these were yperite, lewisite, and irritating agents. Second-generation CWs were composed of nerve agents such as sarin, soman, V agents, and, to a lesser degree, tabun. The development of new third-generation CWs comprised traditional and nontraditional CWs, for example, blister and irritant agents and nerve gases, including new types such as Novichok 5, whose exact chemical structure is unknown, although some assessments have been made (Bajgar, 2006). It could be a nerve agent having high toxicity, and its effects are difficult to treat using common antidotes.

An example of the unintentional use of CWs has also been observed. In March 1968, thousands of dead sheep were discovered in the Skull Valley area in Arizona in the United States. This area was adjacent to the US Army's Dugway open-air testing site for CWs. Nerve gas had drifted out of the test area during aerial spraying and killed the sheep. One year later, on July 8, 1969, the Army announced that 23 US soldiers and one civilian had been exposed to sarin in Okinawa during the clearing of sarin-filled bombs (Sidell et al., 1997).

There are a number of examples of localized conflicts during which CWs have been intentionally used but cannot be verified: from 1951 to 1952 during the Korean War; in 1963 the Egyptians used mustard bombs against Yemeni royalists in the Arabian peninsula; during the Indo-China War (see Vietnam War); in 1970 in Angola, antiplant agents were almost certainly used; and in the former Yugoslavia, there were rumors of the use of psychotomimetic agents.

3.7 Iraq–Iran War and the Afghanistan War

On September 22, 1980, Iraq launched its invasion of Iran. There has been mention of the large-scale use of CWAs in the Iran–Iraq war. In November 1983, Iran informed the United Nations that Iraq was using CWs against Iranian troops. Soon after, the use of CWs was unleashed; in addition, mustard and tabun were used. It is well known that the Iraqi government used these agents against its own citizens, more conspicuously at Halabja in March 1988. This CW attack was the largest against a civilian population in modern times. More than 100,000 Iranians were poisoned with CWAs; sulfur mustard was the most frequently used and induced a number of delayed complications in Iranian veterans (pulmonary, dermal, ocular, immune system depression, reproduction, malignancy, etc.) (Afshari and Balali-Mood, 2006). Other localized conflicts involving alleged use of CWs are described in detail in an extensive review (Robinson, 1971).

The Soviet Union probably used mustard (and nerve gas) in Afghanistan. The Afghanistan War was considered the Soviet Union's "Vietnam." The use of CWs was described by Sidell et al. (1997). The use of CWs by Soviet forces was also significant and has been confirmed

against unprotected subjects. Despite the use of CWs, the withdrawal of Soviet troops from Afghanistan began at the start of 1989.

3.8 Vietnam War

After WWII, the main use of CWs was recorded during 1961–72, when the US Army used defoliants. The herbicide Agent Orange was used during the Vietnam War and led to the injury of more than 1 million Vietnamese and Americans. Agent Orange (a mixture of 2,4-dichlorophenoxy acetic acid and 2,4,5-trichlorophenoxy acetic acid) contained the chemical contaminant dioxin as an impurity that caused many deaths on both sides. There were also other herbicide mixtures, such as Agent White (2,4-D and picloram) and Agent Blue (cacodylic acid). The biological effects of dioxin were described by Sofronov et al. (2001). The first major operation of this type was conducted over the Ca Mau peninsula during September–October 1962. The areas sprayed with defoliants were 5 times larger and 10 times larger in 1965 and 1967, respectively. The scale of the use of defoliants was approximately in proportion to the overall involvement of US troops. In 1970 herbicides and defoliants were used in tens of tons, especially 2,4,5-T. The area sprayed grew from 23 km^2 in 1962 to 22,336 km^2 in 1969. The area exposed to spraying was assessed to be 58,000 km^2 and the number of people exposed was assessed to be more than 1 million; there were more than 1000 deaths. In addition to defoliants used to destroy vegetation concealing the North Vietnamese, the United States used tear gas for clearing tunnels and bunkers. The irritants CS, CN, and DM were reported to have been used. The total CS procured was approximately 7000 tons from 1963 to 1969.

3.9 Development of VX agent

VX was synthesized in the 1960s on the basis of the results of Tammelin and Aquilonius (Tammelin, 1957; Aquilonius et al., 1964). The manufacturing of VX began in the United States in 1961. Construction of the United States' VX agent production plant at Newport, Indiana, was completed in 1961, when the first agent was produced. The production facility only operated for 7 years and was placed on standby in 1968 (Smart, 1997).

During the same period, Soviet scientists developed the so-called Russian VX (VR, RVX, R 033) (Kassa et al., 2006; Kuca et al., 2006). The chemical structure of VX was unknown for a long time. Therefore some attempts to resolve this question have been made (Bajgar, 1968). Because of these ambiguities and difficulties in synthesis, model V agent [EDMM, *O*-ethyl *S*-(2-dimethylaminoethyl) methylphosphonothioate] was initially used in the Eastern Bloc to study antidotal treatment. Another structural analog of VX, known as Chinese VX (CVX), was also developed and studied (Eckert et al., 2006).

A very important step in the development of CWs has been the production of "binary munitions," in which the final stage of synthesis of the agent from precursors is performed in the munition (bomb, shell, or warhead) immediately before or during delivery to the target. In the 1950s, armed forces had begun looking at binary weapons. Until this time, CWs were unitary—the toxic agent was filled in the munition and then stored ready to be used. The binary concept—mixing or storing two less toxic chemicals and creating the nerve agent within the weapon—was safer during storage. The production of binary projectiles began on December 16, 1987, at the Pine Bluff Arsenal in Arkansas.

3.10 Persian Gulf War

On August 2, 1990, Saddam Hussein sent Iraqi troops into Kuwait, allegedly in support of Kuwaiti revolutionaries who had overthrown the emirate. Iraq was known to have a large stockpile of CWs during its conflict with Iran and confirmed that they would use CWs.

President George H.W. Bush ordered US forces to be sent to Saudi Arabia at the request of the Saudi Government (Operation Desert Shield); this was the build-up phase to the Persian Gulf War. As a consequence, in 1996, almost 60,000 veterans of the Persian Gulf War claimed certain medical problems related to their war activities. Some were caused by exposure to nerve agents (released after the bombing and destruction of the sarin production facility). Unexplained "Gulf War syndrome" with low-dose exposure to CWAs was suggested as a possible cause. Extensive research failed to find any single cause of the problem. However, some health effects, including alterations to the immune system 3 months after exposure to low concentrations of sarin, were demonstrated (Kassa et al., 2001, 2003). In the desert, during the autumn and winter of 1990–91, the threat of chemical warfare became very real to allied military personnel. It was demonstrated by the UN Commission that major Iraqi agents included mustard, tabun, sarin, and cyclosarin. Mustard agent was relatively pure, but nerve agents were a complex mixture of the agent and degradation products. During the period from June 1992 to June 1994, the Commission's Chemical Destruction Group destroyed 30 tons of tabun, 70 tons of sarin, and 600 tons of mustard, which were stored in bulk and in munitions.

Suddenly, it became clear to the whole world that there were countries with CWs and biological weapons, and there were other countries that might obtain or produce them.

3.11 Syria

The conflict in Syria has been the most recent conflict in which the use of CWs was confirmed by the UN Mission (UN, 2013). Nerve agent sarin was used in an attack on the Ghouta area of Damascus (August 21, 2013). It is not the intention of this chapter to evaluate political situations; however, it was not possible to decide exactly who used sarin (current government or FSA) against civilian victims. The first complex reactions were published in October 2013 in the CBRNe World (Higgins, 2013; Johnson, 2013; Kaszeta, 2013; Winfield, 2013). For the Mission, these were not ideal conditions: difficult political situation, chaotic scene, and timing that was not ideal. However, the report was well-structured and conclusions were clear: sarin was present in some samples and rocket remains, and selected survivors showed symptoms supporting sarin exposure (Johnson, 2013).

There are different data regarding the number of victims, initially varying from hundreds to thousands. The Syrian Observatory for Human Rights reported more than 500 deaths and thousands of patients displaying "neurotoxic symptoms," including civilians and children. Medicine Sans Frontiers said at least 3600 patients had these symptoms and, of those patients, 355 had died. The UN Mission selected 36 of 80 survivors who met the criteria established by the Mission. Symptoms consistent with organophosphate intoxication were observed: decreased consciousness (78%), dyspnea (61%), blurred vision (42%), eye irritation or inflammation (22%), lacrimation (8%), miosis (14%), salivation (22%), vomiting (22%), and convulsion (19%). Johnson (2013) did not mention the postmortem samples or data regarding dead persons. The treatment of victims and the course of poisoning, including laboratory results, have not been specified. However, laboratory examinations would be useful, as in the case of Tokyo victims (Polhuis et al., 1997). It would be possible to use other methods of laboratory diagnoses of nerve agent intoxication, as described previously (Noort et al., 2009; Schans van der and Gupta, 2009; Bajgar, 2013). Autopsies of victims were not conducted but would have been useful, as would postmortem examinations of dead animals. Regarding CWs in Syria, they will be destroyed under the supervision of the Organization for Prohibition of Chemical Weapons (OPCW) (for Syria, CWC entered into force on October 14, 2013) and with international assistance.

3.12 Unintentional use of toxic chemicals

There have been two main accidents connected with the release of toxic chemicals. In July 1976, in Seveso, Italy, more than 40,000 people were exposed to dioxin, a persistent and highly toxic chemical. The first signs were skin lesions appearing on children, and after some months there was evidence of chloracne. Health consequences have been observed from that time to the present. The Seveso accident was possibly the most systematically studied dioxin contamination incident. A similar contamination of one building of the Spolana company in Neratovice (a town in the former Czechoslovakia) was also observed (Bajgar et al., 2007a; Pelclová et al., 2011). Another example, the Bhopal accident, is probably the greatest industrial disaster in history. In 1984 on December 2 and 3, water inadvertently entered the methylisocyanate storage tank (containing approximately 40 tons of this chemical). As a result, methylisocyanate was released into the surrounding area. There was no warning. Many people who inhaled high concentrations of toxic gas asphyxiated because of extensive lung damage. Approximately 150,000 people were intoxicated (50,000 seriously poisoned) and more than 2500 people died (Bajgar, 2006).

3.13 Terrorist use of chemical weapons

Terrorists have expressed an interest in nerve agents and have deployed them in attacks on unprotected civilians (Rotenberg and Newmark, 2003). A Japanese religious cult, Aum Shinrikyo, independently manufactured numerous chemical and biological agents. The first such attack with sarin occurred in Matsumoto in 1994 and in the Tokyo subway in 1995. Thousands of people were affected and dozens of people died (Ohtomi et al., 1996; Nagao et al., 1997; Okomura et al., 1998; Yokoyama et al., 1998). In Matsumoto (1994), 600 people were poisoned and hospitalized, and seven died (Morita et al., 1995; Nakajima et al., 1997; Yoshida, 1994). The attack in the Tokyo subway (1995) resulted in 5500 people seeking hospital evaluation and 12 deaths (Bajgar, 2006). An interesting terrorist act was described by Tsuchihashi et al. (2005)—a fatal intoxication with VX administered percutaneously.

Nerve agents belong to the group of OPs. These compounds in the form of pesticides are commercially available and are used in agriculture, which can lead to professional, suicidal, or accidental intoxication. The mechanism of action, diagnosis, and treatment of intoxication with OP pesticides and nerve agents are very hot topics at present. Moreover, some principles of the effects, diagnosis, and therapy are very similar for OP and highly toxic nerve agents; therefore the principle of action and effective treatment can also be applied in general for the civilian sector.

The use of these chemicals was observed in Moscow in 2002. A Moscow theater hostage crisis resulted in the seizure of a crowded theater on October 23, 2002, by approximately 40 armed Chechen militants who claimed

allegiance to the separatist movement in Chechnya. They took 850 hostages and demanded the withdrawal of Russians from Chechnya and an end to the war in Chechnya. The leader of the terrorists was 22-year-old Movsar Baraev. After 2.5 days of waiting, Russian forces used an unknown gas that was pumped into the ventilation system. Officially, 39 terrorists and at least 129 of the hostages (nine of them foreigners) were killed. Some estimates have put the civilian death toll at more than 200. It was thought that the security services used an aerosol of a CWA, first assessed as BZ, but later it was specified that an aerosol anesthetic of the fentanyl type was used (Bajgar and Fusek, 2006).

In hospitals, the survivors were cut off from any communication with the outside world and their relatives were not allowed to visit them. An incorrect list of hospitals for victims was released. The main problem was the lack of information about those dealing with the identification and characterization of the chemical used and the unavailability of known antidotes (e.g., naloxon) by medical staff treating the victims (Bajgar et al., 2007a). It appeared from this event that there were compounds not explicitly enumerated in the CWC and therefore not controlled by this Convention. Fentanyl can be considered as a nonlethal weapon (a group of so-called calmatives) and these chemicals can also be used to incapacitate animals; of course, its use against humans is not excluded (Bajgar, 2006; Hess et al., 2005).

3.14 Negotiations

Although the Cold War was continuing, the political situation led to increased activities in international negotiations. At the Conference on Disarmament in Geneva, some attempts to negotiate a ban of CWs were begun, first as an ad hoc Working Group, and later as an ad hoc Committee on Chemical Weapons with the mandate to negotiate the text of a convention banning CWs.

The discussions in Geneva were more intensive from 1987 and, in 1992, the elaboration of the so-called rolling text of future CWCs was finished. During these negotiations, the text of future Conventions ("rolling text") was enlarged: the final report (CD/342) of February 2, 1983, contained 23 pages; the same report of August 23, 1985 (CD/636), had 46 pages; and CD/952 of August 18, 1989, contained 134 pages. Simultaneously with the Geneva negotiations, in September 1989, the Memorandum of Understanding between the Governments of the United States and the USSR regarding a bilateral verification experiment and data exchange related to the prohibition of CWs, otherwise known as the Wyoming Meeting, started negotiations between the two main possessors of CWs. These countries also contributed to the negotiations in Geneva: they demonstrated their CWs to the Conference on Disarmament in the United States in November 1986 (Tooele) and in the USSR in October 1987 (Shikhany). The final document of the Convention is approximately 200 printed pages. The Convention was then agreed on in New York at the UN General Assembly and signed in Paris in 1993. The CWC (Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and on their Destruction) entered into force on April 29, 1997, 180 days after the deposit of the 65th instrument of ratification of the Convention by Hungary. At this time, 87 countries ratified the CWC and became original state parties to the Convention. Simultaneously, the OPCW in The Hague started its work of supervising the destruction of CW stocks and monitoring the world's chemical industry to prevent future misuse. There are many activities of the OPCW, for example, training of inspectors for control of destruction of CWs including their medical protection, research, and supported activities, solving problems due to practical implementation of the CWC, control of chemical and military facilities, and other activities. Russia and the United States were unlikely to meet the final stockpile destruction deadline of April 29, 2012. By the middle of 2008, 183 signing states and 194 recognizing states had adhered to the Convention (Davey, 2008). However, there are still states that are nonsignatories to the Convention. CWs have a long and ancient history. A lack of CW use in WWII suggested that "gas warfare" had ended. However, further development and the utility of chemicals in Vietnam and in terrorist attacks have maintained the military interest in CWs.

Current information of OPCW provides the status of the destruction of CWs. April 29, 2012, was suggested to be the prolonged period for CW destruction. Seven state parties declared they possessed CWs (Albania, India, Iraq, South Korea, Libya, Russia, and the United States). The stocks of Albania, India, and South Korea were destroyed. To this date, 73.72% of all declared CWs (a total of 71,195.086 tons) had been destroyed (Streda, 2013). On the basis of the Conference of the State Parties (16th Session, December 2011), the destruction period was prolonged for Russia (2015), Libya (2016), and the United States (2023). Simultaneously, CW-producing facilities were also destroyed or dismantled—13 state parties declared 70 of these facilities (Bosnia and Herzegovina, China, France, India, Iraq, Iran, Japan, South Korea, Libya, Russia, Serbia, Great Britain, and the United States) and 43 of these objects were destroyed and 21 were dismantled for peaceful purposes.

It is clear that the use (incidental or otherwise) of toxic chemicals has impacts on different spheres of human existence, such as state structures and infrastructure, economics, psychic and public behavior, and the environment. Toxic chemicals are a great consumer of

natural sources, both renewable and nonrenewable. They also consume raw materials and energy and, as a consequence, cause pollution of the environment and lead to a deficiency of raw materials throughout the world and therefore an unequal distribution of the world's natural sources. The impact on the psychology of humankind is also important, following either chemical wars (both global and local) or use of these chemicals by terrorists. The development of new technologies is equally important because they influence, positively and negatively, further human development. Research in this direction not only can contribute to "improvement" of chemicals to obtain more effective CWAs but also can improve our knowledge of basic sciences (toxicology, neuropharmacology, etc.) and allow us to better understand physiological functions in general. It is appropriate to recall the history of cholinesterases and their inhibitors. The existence of cholinesterases was predicted by H.H. Dale in 1914, 14 years before acetylcholine was demonstrated as a natural constituent of animal tissues. This research approach was changed during WWII and cholinesterases acquired a special significance in the context of chemical warfare and nerve agents (Silver, 1974). Another publication in this area (Koelle, 1963) can be considered as the first to deal with anticholinesterase agents including CWAs—nerve agents. One can only hope that in the future the only physiological and pharmacological research will be performed in a nonmilitary framework, but that may not be the case.

3.15 Concluding remarks and future directions

The threat of the use (either military or terrorist) of CWAs (and other toxic chemicals) still exists. The military use of these agents is limited, but their terrorist use is unlimited. The spectrum of these agents is very large and the ability to be prepared against the use of toxic chemicals is necessary.

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Chapter 4

Sarin attacks in Japan: acute and delayed health effects in survivors

Chapter 4.1

Part 1 Sarin attacks in Japan: acute and delayed health effects in survivors of the Matsumoto incident

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4.1.1 Introduction

Sarin, isopropyl methylphosphonofluoridate, is an organophosphate agent which is highly toxic and considered to be a lethal chemical warfare agent. It was infamously used against the Kurdish community in Iraq, which was substantiated by analysis of samples obtained from ruptured munitions from the mountainous region of northern Iraq (Black et al., 1994; Black, 2009).

4.1.2 Matsumoto sarin incident

On the night of June 27, 1994, 9 months before the Tokyo subway incident, a terrorist released 12 L of high-purity sarin vapor in a residential area of Matsumoto City, Japan, using a heater and fan and a remodeled truck (Nakajima et al., 1998a; Morita et al., 1995). It was rainy with a 0.6−1.8 m/s wind from the southeast in the area. Seven persons who lived near the district where sarin was released died, with another dying later, but many victims were hospitalized and consulted doctors. Common features among these victims were a decrease in miosis and decreased serum acetylcholine esterase (S-AChE) level, which are symptoms often observed in exposure to organophosphorus chemicals. Six days after the incident, the causative agent was reported to be sarin.

Questionnaire surveys were conducted for 2052 persons living around the sarin-released area and rescue team 3 weeks after the sarin incident (Nakajima et al., 1997, 1998a). There were 1743 respondents (84.9%), and the follow-up questionnaire study for this cohort was conducted at 4 months, 1, 3, and 5 years after the sarin incident (Nakajima et al., 1999). The first study revealed that 76% of sarin victims were inhabitants in the block separated by postal code where sarin was released; the prevalence was broken down by region with 44%−47% in the north, 72% in the northeast, 47% in the east, and 35% in the south locations. There were an estimated 600 victims, including eight who died (five men and three women). All victims were distributed in an area of 800 m north to south and 570 m east to west, including the point of sarin release. The majority (80%) of victims were located in an elliptical area with a 400-m long axis northeast of the site. All eight persons who died, and all hospitalized victims, were located in this area. Thus, sarin was distributed according to the wind direction. Other victims were distributed outside of the elliptical area, but experienced only minimal symptoms not requiring medical consultation.

Distribution by the time of onset of sarin symptoms was bimodal: the first peak was between 11:00 and 12:00 p.m. on the 27th of June, and the second peak was between 8:00 and 9:00 a.m. the next day (Fig. 4.1.1). Compared to the second peak, sarin victims who felt the symptoms during the first peak were younger and with more severe symptoms, all required hospitalization, a higher percentage were outpatients, and all lived in the elliptical area with a 400-m long axis. Victims in the second peak did not feel severe symptoms like the hospitalized ones, but no significant difference was observed between the numbers of victims and the distance of their home from the site where sarin was released.

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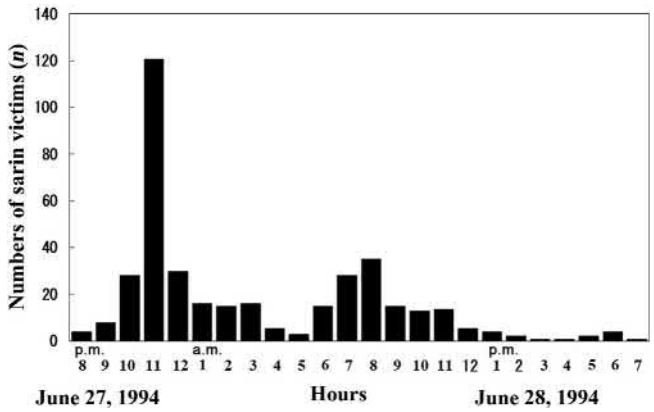

FIGURE 4.1.1 Distribution by time of the symptoms onset in sarin-exposed victims. Abscissa axis represents the time of symptom onset and spindle axes represents the number of victims reporting first onset of symptoms.

One month after the sarin incident, a medical checkup survey was conducted for applicants (155 persons: 68 men and 87 women) who requested it in the first questionnaire survey. In this survey, S- and E-AChE activities were also measured. The pupil diameter in sarin victims was smaller compared with persons who had not suffered from sarin intoxication, even 4 weeks after exposure. S- and E-AChE activities were also lower in sarin victims than nonvictims. Significant differences were also observed in both test results among inpatients, outpatients, and patients without a medical consultation. Therefore, pupil diameter and AChE activity, especially E-AChE activity, are excellent biomarkers to determine the severity of sarin poisoning.

4.1.3 Acute impacts

In the early stage after the sarin attack, victims felt symptoms which disturbed the nose and respiratory organs, such as sneezing, rhinorrhea, nasal voice, sore throat, coughing, dyspnea, and eye abnormalities such as darkness and narrowing of the visual field, flickering of vision, ocular pain, increased lacrimation, blurriness of vision, and diplopia (Fig. 4.1.2). These symptoms were caused by the muscarinic effects of sarin. In addition to these symptoms, the more severely affected victims were inpatients who felt headache, nausea, vomiting, dizziness, dysesthesia of the extremities, muscle cramp, gait disturbance, paresis of perioral muscle, dysphagia, and fatigue. The symptoms of dysesthesia of extremities, gait disturbance, and fatigue were reported to be caused by the nicotinic effects of sarin (Sidell, 1974).

The frequency of early symptoms associated with sarin poisoning decreased over time. Three weeks after the exposure, symptoms caused by muscarinic effects of sarin quickly resolved, and the most prominent complaint was dysesthesia of the extremities, suggesting that sarin affects the peripheral nervous system. However, this symptom was not observed 4 months after exposure, and none of the victims showed any abnormalities in nerve conduction studies. Symptoms reported immediately after sarin intoxication in victims resolved 4 months later. Contrary to these phenomena, the complaint of asthenopia began to occur in victims with lowered E-AChE activity at 4 weeks after intoxication. It is noteworthy that the frequencies of this complaint further increased at 4 months after intoxication and it was still present after 1 year, although E-AChE activity had returned to normal levels. The symptoms of fatigue increased again and new reports of asthenia rose after 1 year.

4.1.4 Long-lasting complaints

A questionnaire survey of sarin exposure-related symptoms was conducted for the first survey cohort at 3 ($n = 1237$, 60.3%; victims = 318; nonvictims = 919) and 5 years after the sarin incident ($n = 838$, 40.8%; victims = 169; nonvictims = 669) (Nakajima et al., 1999). E-AChE activity 4 weeks after the sarin incident was compared between persons who had and did not have symptoms associated with sarin exposure after 1 year. The E-AChE activity in those exhibiting symptoms of fatigue, asthenia, and asthenopia were lower than those who were asymptomatic; this suggests that these symptoms were observed in persons who had more severe sarin intoxication. The locations of subjects who were symptomatic 1 year after sarin exposure were mapped by postal code. Almost all symptomatic people were located in an elliptical area with a 400-m axis northeast of the sarin release site.

Three years after the sarin incident, 46 (27.2%) victims had one or more symptoms, whereas only 36 (5.4%) nonvictims had one or more symptoms, indicating that there were more symptomatic individuals in the victim

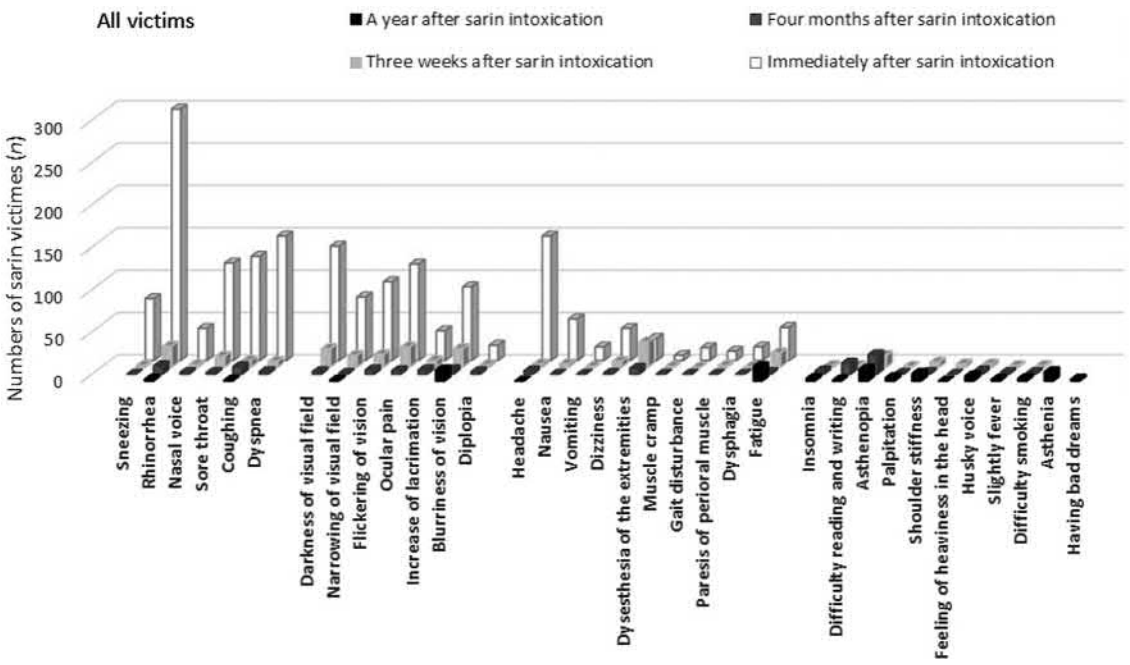

FIGURE 4.1.2 Transition of each symptom immediately, at 3 weeks, at 4 months, and 1 year after sarin exposure. The vertical axis shows the number of people who had each symptom.

group. Of these victims, 40 (24.0%) complained of asthenopia, 25 (15.0%) complained of fatigue, 18 (10.8%) complained of blurred vision, 15 (9.0%) complained of shoulder stiffness, and 14 (8.4%) complained of asthenia and headache. Conversely, among the nonvictims, 21 (3.1%) complained of asthenopia, 22 (3.3%) complained of fatigue, 13 (1.9%) complained of blurred vision, 25 (3.7%) complained of shoulder stiffness, 11 (1.8%) complained of asthenia, and 7 (1.0%) complained of headache. The percentages of these symptoms were greater in victims than in nonvictims. The percentage of victims having slight fever was slightly greater than that of nonvictims. In agreement with these findings, the odds ratios of these symptoms were greater in the victim group given their exposure to sarin. No significant differences between nonvictims and victims were noted in symptoms of bad dreams, insomnia, narrowing of the visual field, difficulty in smoking, husky voice, and palpitations. The odds ratios for each symptom after standardizing for age did not differ from those mentioned above.

The prevalence of victims with symptoms both 1 and 3 years after the sarin incident (victims with consistent symptoms) was compared with that of nonvictims. Significant differences were observed in the prevalence of symptomatic subjects or those with symptoms of blurred vision and asthenopia between victims and nonvictims. Similarly, odds ratios for symptomatic subjects or those with both symptoms were also higher in the victims than in nonvictims.

Taken together, symptoms of asthenopia, fatigue, blurred vision, shoulder stiffness, asthenia, and headache are later-manifested sequelae of sarin poisoning because (1) these complaints broke out in a dose-dependent manner; (2) the prevalence of these symptoms was significantly greater in victims compared with nonvictims; and (3) some of these complaints persisted after sarin exposure until 5 years, with the odds ratios of these symptoms clearly higher in the victim group.

In the additional medical checkup survey and clinical investigation conducted 3 years after the incident, seven of 31 admitted patients were diagnosed with sequelae of sarin: four showed epileptic electroencephalographic changes, three showed arrhythmia and sensory polyneuropathy with reducing sensory nerve conduction velocity, and one continued to have visual field constriction at a year postexposure, but 2 years after the poisoning, this was resolved completely (Sekijima et al., 1997). These victims responded to every questionnaire survey with complaints of asthenopia, fatigue, asthenia, blurriness of vision, headache, and/or slight fever.

The urinary sarin metabolites isopropylmethylphosphonic acid (iPMPA) and methylphosphonic acid (MPA) were measured in an unconscious severely ill victim who was brought into the hospital (Nakajima et al., 1998b). iPMPA was detected until 156 h after sarin exposure, but MPA was not detected at that time point. Therefore, this suggested that sarin metabolites were excreted into the urine within 1 week, even in those severely affected. The total excretion

amounts of iPMPA and MPA were 2.1 mg and 0.45 mg, respectively. When all the sarin inhaled was excreted within a week (as determined by these two metabolites), the victim was considered to have been exposed to 2.79 mg (0.05 mg/kg) sarin during the incident. This shows that sarin inhaled by humans is quickly metabolized, and this chemical and its metabolites are excreted from the body within 1 week, even in lethal doses. The later sequelae may be due to irreversible effects of acute exposure and chronic exposure to the chemical or its metabolites.

4.1.5 Psychological impacts

Psychological problems associated with posttraumatic stress disorder (PTSD) have been noted after accidents such as the Tokyo subway sarin poisoning incident (Ohbu et al., 1997) and earthquakes (Najarian et al., 1996; Goenjian et al., 1997). Five years after the sarin incident in Matsumoto, psychiatric symptoms were investigated for the follow-up cohort using a questionnaire survey at the same time as the later sequelae investigation of sarin intoxication. The questionnaire consisted of three clusters: (1) arousal ("sleep disturbances," "strong fear," "unease and frustration," and/or "lack of concentration, frequent errors, and mistakes"); (2) re-experiencing ("distressing dreams," "flashbacks to the sarin attack," and/or "distressing reminders when approaching the sarin attack site"); and (3) avoidance ("avoidance of sarin attack-related news and/or conversation," "apathy," "poor memory," "loss of memory toughness, becoming depressed," and/or "physical tension such as shoulder stiffness and sweaty hands"). In the survey, a person with one or more symptomatic subjects in each cluster was judged to have "symptomatic arousal," "symptomatic re-experience," and "symptomatic avoidance." There was no difference noted in PTSD-related symptoms between male victims and nonvictims. However, the odds ratios of symptomatic arousal, re-experiencing, and avoidance were significantly higher in female victims compared with female nonvictims.

4.1.6 Ten years after the sarin incident

The final questionnaire survey was conducted 10 years after the sarin incident. The questionnaire was distributed to 1813 inhabitants, which was similar to the follow-up cohort, but differed slightly because of a population inflow and outflow phenomenon. In total there were 727 (40.1%) responses. Of these responses, 668 persons agreed to complete the survey. There were 511 valid responses to the question regarding if the respondent felt sarin-related symptoms. There were 99 (19.4%) original Matsumoto sarin victims from 10 years previously and 412 (80.6%) nonvictims. The crude odds ratio (95% confidence interval, CI), unadjusted for age and sex, of symptoms of asthenopia at a slight-intermediate level for all victims was 4.11 (2.26−7.49), and that at considerable to very high levels was 7.22 (3.35−15.57), that of fatigue was 2.40 (1.41−4.07) and 4.45 (2.10−9.43), that of blurred vision was 2.54 (1.48−4.37) and 3.93 (1.74−9.00), that of asthenia was 2.63 (1.58−4.36), and 2.66 (1.01−7.02) and that of headache was 2.61 (1.49−4.57) and 3.32 (1.01−10.93), respectively (Table 4.1.1) (Report on health surveys of the Matsumoto sarin attack, 2015). It was obvious that many victims still had complains of eye abnormalities, especially "asthenopia" and "blurred vision." However, there was a significant difference between victims and nonvictims in other awareness observed as sequelae after 5 years.

With regard to PTSD symptoms, although a cluster analysis was not conducted in the 10-year survey, the odds ratio of each symptom was analyzed. In female victims, crude odds ratios (CI) of "sleep disturbance," "distressing dream," "flashbacks to the sarin attack," "unease and frustration," "lack of concentration, frequent errors and mistakes," "apathy," and "physical tension such as shoulder stiffness and sweaty hands" were 2.35 (1.14−4.86), 2.50 (1.09−5.73), 5.35 (2.49−11.50), 2.70 (1.13−6.41), 2.29 (1.06−4.98), 2.47 (1.10−5.41), and 2.18 (1.00−4.75), respectively, at slight-intermediate levels (Table 4.1.2). In male victims, the odds ratio of "sleep disturbance," "flashbacks to the sarin attack," "unease and frustration," "loss of mental toughness, becoming depressed," and "physical tension such as shoulder stiffness and sweaty hands" were 3.39 (1.44−7.72), 3.76 (1.49−9.49), 4.92 (1.59−15.73), 2.35 (1.04−5.30), and 2.44 (1.08−5.50), respectively, at slight-intermediate levels. In contrast, frequencies of symptoms of "distressing reminders when approaching the sarin attack site" and "poor memory" were significantly higher in female victims than female nonvictims, and the odd ratios (95% CI) both at slight-intermediate levels and at considerable to very high levels were 3.77 (1.72−8.27) and 6.72 (1.07−42.21), and 2.42 (1.16−5.04) and 4.58 (1.23−17.10), respectively. No such findings were observed in male victims. The PTSD symptoms observed at considerable to very high levels alone were "avoidance of sarin attack-related news and/or conversations" (odds ratio, CI: 15.13, 1.63−140.05) and "loss of mental toughness, becoming depressed" (3.76, 1.01−14.02) in female victims; "distressing dream" (13.56, 1.36−135.37), "avoidance of sarin attack-related news and/or conversation" (14.08, 1.41−140.75), and "poor memory" (3.07, 1.04−9.08) were observed in male victims. Thus, the psychological impact of sarin appears to be generally stronger in women than in men, which is similar to the effects on PTSD found after 5 years. The odds ratio of "avoidance of sarin attack-related news and/or conversation" was highest in both men and women at considerable to very high levels.

TABLE 4.1.1 **Typical subjective symptoms in sarin victims and nonvictims (all responders) at 10 years after the sarin incident.**

	Symptom severity	Victims	Nonvictims	Odd ratio (95% CI)
Asthenopia	No	18	156	Ref
	Slight-intermediate levels	47	99	4.11 (2.26–7.49)
	Considerable to very high levels	20	24	7.22 (3.35–15.57)
Fatigue	No	32	187	Ref
	Slight-intermediate levels	39	95	2.40 (1.41–4.07)
	Considerable to very high levels	16	21	4.45 (2.10–9.43)
Blurred vision	No	32	178	Ref
	Slight-intermediate levels	37	81	2.54 (1.48–4.37)
	Considerable to very high levels	12	17	3.93 (1.71–9.00)
Asthenia	No	38	202	Ref
	Slight-intermediate levels	42	85	2.63 (1.58–4.36)
	Considerable to very high levels	7	14	2.66 (1.01–7.02)
Headache	No	46	214	Ref
	Slight-intermediate levels	28	50	2.61 (1.49–4.57)
	Considerable to very high levels	5	7	3.32 (1.01–10.93)

CI, Confidence interval; *Ref*, reference.

TABLE 4.1.2 **Typical subjective psychological symptoms in sarin victims and nonvictims (women only) at 10 years after the sarin incident.**

Symptoms	Symptom severity	Victims	Nonvictims	Odd ratio (95% CI)
Sleep disturbance	No	26	102	Ref
	Slight-intermediate levels	18	30	2.35 (1.14–4.86)
	Considerable to very high levels	3	7	1.68 (0.41–6.95)
Distressing dream	No	35	124	Ref
	Slight-intermediate levels	12	17	2.5 (1.09–5.73)
	Considerable to very high levels	1	0	
Flashbacks to the sarin attack	No	24	122	Ref
	Slight-intermediate levels	20	19	5.35 (2.49–11.50)
	Considerable to very high levels	3	0	
Unease and frustration	No	34	125	Ref
	Slight-intermediate levels	11	15	2.70 (1.13–6.41)
	Considerable to very high levels	1	1	3.68 (0.22- 60-31)

(*Continued*)

TABLE 4.1.2 (Continued)

Symptoms	Symptom severity	Victims	Nonvictims	Odd ratio (95% CI)
Lack of concentration, frequent errors and mistakes	No	29	114	Ref
	Slight-intermediate levels	14	24	2.29 (1.06–4.98)
	Considerable to very high levels	3	3	3.93 (0.75–20.50)
Apathy	No	29	110	Ref
	Slight-intermediate levels	13	20	2.47 (1.10–5.54)
	Considerable to very high levels	2	5	1.52 (0.28–8.22)
Physical tension such as shoulder stiffness and sweaty hands	No	27	105	Ref
	Slight-intermediate levels	14	25	2.18 (1.00–4.75)
	Considerable to very high levels	4	10	1.56 (0.45–5.34)
Distressing reminders when approaching the sarin attack site	No	27	121	Ref
	Slight-intermediate levels	16	19	3.77 (1.72–8.27)
	Considerable to very high levels	3	2	6.72 (1.07–42.21)
Poor memory	No	14	77	Ref
	Slight-intermediate levels	26	59	2.42 (1.16–5.04)
	Considerable to very high levels	5	6	4.58 (1.23–17.10)
Avoidance of sarin attack-related news and/or conversations	No	32	121	Ref
	Slight-intermediate levels	12	21	2.16 (0.96–4.85)
	Considerable to very high levels	4	1	15.13 (1.63–140.05)
Loss of mental toughness, becoming depressed	No	25	94	Ref
	Slight-intermediate levels	15	41	1.38 (0.66–2.88)
	Considerable to very high levels	5	5	3.76 (1.01–14.02)

CI, Confidence interval; *Ref*, reference.

The symptoms reported in the 10-year survey were cross-referenced to receiving medical consultation: whether he/she was an inpatient, outpatient, or other. The crude odds ratios of "asthenopia," "unease and frustration," and "loss of mental toughness, becoming depressed" were significantly higher in inpatients and outpatients compared with the others group.

Ten years after the attack, many victims still complained of physical and psychological disorders. Many of these symptoms were dependent on the severity of sarin poisoning at immediately after the attack.

4.1.7 Conclusion

Sarin exposure acutely affected the human body with so-called muscarinic and nicotinic effects, and also caused long-term effects on physical and mental status. Symptoms of asthenopia, fatigue, blurred vision, shoulder stiffness, asthenia, and headache were manifested as later sequelae. In particular, the crude odds ratio of asthenopia was extremely high compared with other complaints. Psychological disorders were also observed 10 years after sarin intoxication. The impact of sarin on psychological disorders was greater in women than men.

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Chapter 4.2

Part 2 Tokyo sarin attack: acute health effects

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4.2.1 Overview of the Tokyo subway sarin attack

This attack took place during the morning rush hour, at about 8 a.m. on March 20, 1995, the day before the Spring Equinox holiday. The attack was carried out by members of a cult known as Aum Shinrikyo to distract police from carrying out a raid on the group's headquarters. The terrorist target was government buildings in Kasumigaseki in the heart of Tokyo. Most offices in Kasumigaseki open for business at 9:30 a.m., but the early-morning rush hour was unusually heavy because it was a Monday. Some believe that the time of 8 a.m. was chosen because some cult members had inside information about the government offices. Police suspected, based on an undercover investigation that they were conducting, that Aum Shinrikyo was manufacturing sarin for use in a terror attack, but few people, even within the police department, were aware of this fact. The police did not have personal protective equipment (PPE), which meant that they had to borrow PPE and receive training on use of the equipment from the Self-Defense Forces. Members of the Self-Defense Forces were alerted to some of Aum Shinrikyo's planned activities, but the general public, including healthcare providers and fire department personnel, knew nothing of these activities (Fig. 4.2.1). According to a subsequent police report, the terrorists placed sarin in five subway trains in the following way.

Approximately 600 g of sarin at a concentration of 33% was mixed with hexane and *N*,*N*-diethylaniline and placed in a nylon/polyethylene bag. Five terrorists then wrapped the bags in newspaper, punctured the bags with the tips of their umbrellas, and left the bags on the trains. In this way, the sarin seeped out of the bags and vaporized, but no other active means of dispersal were used. In this sense, as well as the relatively low number of deaths, the Tokyo subway sarin attack was not considered a full-scale attack.

Of the bags of sarin used in the attack, two bags were not punctured. These bags were returned to the police laboratory for analysis. At Kasumigaseki, one of the subway stations on the Chiyoda subway line, two station employees collapsed and died on the platform after they cleaned and removed one of the bags that didn't get punctured, even though they were wearing gloves. The number of victims of this attack varies depending on the source, but all known information confirms that 13 people died in the attack, and it is generally believed that at least 5500 victims suffered mild to serious injuries. Firefighting agencies estimate 5642 victims, and the police, 3796 victims, while official figures released by the subway company put the total number of victims at 5654. This includes the 12 who died (10 passengers, 2 employees), those hospitalized (960 passengers, 39 employees), and those treated for minor injuries (4446 passengers, 197 employees).

Thus, the way in which we use the lessons learned from this attack will affect our ability to deal adequately with future terrorist attacks using sarin, which could be even greater and more serious with respect to the number of victims. Can we really assume that only 12 of the approximately 5500 victims died because the Japanese

FIGURE 4.2.1 Scene from a sarin attack at Tsukiji station.

medical system was particularly well prepared for such an eventuality? Probably not. It is more likely that the relatively small number of fatalities was due to the low concentration of sarin and the passive means of dispersing it. From this perspective, the Matsumoto sarin attack 1 year earlier was more aggressive than the Tokyo subway sarin attack. In a trial after the Matsumoto incident, it was revealed that a 70% concentration of sarin was actively volatilized using an electric heater and dispersed using an electric fan. A total of seven victims died and 660 were injured, and one victim died 14 years after sarin exposure. In other words, if the Tokyo subway sarin attack had been conducted using the same means as those employed in the Matsumoto sarin attack, the number of fatalities may have been 50 or 60. So humanity has not yet experienced the effects of a full-scale sarin attack in a major city.

Even if it did not rise to the level of a major attack, this incident was the first chemical terrorist attack in a large city. There were few first-responders who could even have conceived of such an attack, let alone be prepared to rapidly evacuate victims from the subway stations. Many passengers who had difficulty walking rushed out of the trains and onto the subway platform and fell down, which would have increased their exposure to the sarin permeating the stations. In addition, the site to which many of the victims were finally evacuated at ground level, where they could lie down, was close to an air exhaust vent from the subway below, so the exposure continued.

The first call for an ambulance came 9 min after the 8 a.m. attack, with the first report of a "victim with seizures at Kayabacho Station." By 8:15 a.m., the reports of victims started to increase. Around this time, the fire department received a report from Tsukiji Station stating that "an explosion occurred and several people were injured." Calls for ambulances eventually came from 19 subway stations, and after 8:30 a.m., victims began to pour into local clinics and hospitals. According to the Tokyo Fire Department, 5493 people were treated at 267 medical institutions in Tokyo, and 17 people were treated at 11 medical institutions outside Tokyo. Among the victims, 53 were seriously injured (Ieki, 1997). Another source states that a total of 6185 people were treated at 294 medical institutions (Chigusa, 1995). The discrepancy in the number of victims reported by different agencies attests to some of the confusion at the time. St. Luke's Hospital received the largest number of victims (640 on the day of the attack), probably because of its close proximity to the Hibiya line, where a large number of victims were located, and because of a report on television stating that "St. Luke's Hospital has the antidote for treatment."

4.2.2 Emergency treatment of sarin toxicity

The standard treatment for sarin toxicity includes (1) maintaining the airway, (2) assisting breathing, and (3) supporting circulation. Endotracheal intubation was performed frequently in victims of the Tokyo subway sarin attack. However, in the Matsumoto sarin attack, endotracheal intubation was more difficult to carry out in many victims because of airway hypersecretion and bronchospasm. This difference in symptoms is attributable to the 70% concentration and the active means by which the sarin was dispersed at Matsumoto, as opposed to the

much lower 33% concentration and passive means of dispersal employed in Tokyo. Dr. Frederick Sidell, an expert on chemical terrorism in the United States, advocated decontamination, drugs, airway, breathing, and circulation (DDABC) as the basic treatment for nerve agent poisoning. Even if the advised emergency treatment is followed, initial efforts to achieve adequate ventilation may be in vain. Efforts to achieve adequate ventilation should be made after at least initial administration of atropine to control the buildup of airway secretions and bronchoconstriction (Sidell, 1997). If healthcare professionals learn from the Matsumoto attack, they can better recognize early parasympathetic nervous symptoms, including miosis, hypersecretion, and rhinorrhea, as common indications of chemical terrorism due to nerve agents, and will therefore be able to institute appropriate treatment with antidotes in time. In large-scale disasters with many victims, treatment is often deferred in those with cardiopulmonary arrest (CPA; so-called black tag). However, at St. Luke's Hospital, one in three persons with CPA and two patients with respiratory arrest made a full recovery and were discharged. This high rate of recovery and return to the community is unlike that seen in other types of disasters. Therefore, if medical resources are available, all victims of a sarin attack should be aggressively treated, including cardiopulmonary resuscitation (CPR) when necessary.

The global standard for the treatment of sarin toxicity is the administration of (1) atropine, (2) an oxime agent like 2-PAM, and (3) diazepam (Medical Letter, 2002).

Recommended doses of atropine are 2 mg in patients with mild symptoms that are primarily ocular, but without respiratory symptoms or seizures; 4 mg in patients with moderate symptoms, including respiratory symptoms such as dyspnea; and 6 mg in patients with severe symptoms, including seizures and respiratory arrest. The standard administration route should be intramuscular. As mentioned previously, intravenous administration of atropine in the treatment of severe symptoms such as hypoxemia can induce ventricular fibrillation; thus, intramuscular administration is advised. Oxime agents, such as 2-pralidoxime methiodide (2-PAM) or 2-formyl-1-methylpyridinium iodide oxime, should also be given. The recommended dose for 2-PAM in moderate and severe cases of inhalation, or for liquid exposure to a nerve agent, is 1 g by intravenous infusion over 20–30 min. Further continuous administration of 500 mg/h may also be required in severe cases. Since the rate of aging of the nerve agent–enzyme bond is correlated with time until 2-PAM is administered, if the aging half-life of sarin is 5 h, then 2-PAM must be administered before this time. The oxime of choice for sarin and VX is 2-PAM, but asoxime chloride (HI-6) should be used for soman and obidoxime for tabun. Seizures are treated with diazepam. This three-drug combination (atropine, 2-PAM, and diazepam) is the global recommendation for sarin toxicity, and autoinjectors are available in several countries (Vale et al., 2006) (Fig. 4.2.2).

After the Tokyo subway sarin attack, St. Luke's Hospital, which treated 640 victims, used about 700 ampules of 2-PAM and 2800 ampules of atropine (Okumura et al., 1998). This calculates out to 550 mg of 2-PAM and 2.2 mg of atropine per victim. The route of administration was intravenous in all cases, with a total dose of 1.5–9 mg of atropine in severe cases (Okumura

FIGURE 4.2.2 Sarin victims at St. Luke's International Hospital.

et al., 1996); this range of dose reflects the low concentration and passive means of sarin dispersal used in the Tokyo attack.

However, in Tokyo, no one was saved by administration of 2-PAM; conversely, no one died because they did not receive it. In other words, if the victims' survival was the ultimate goal, there was no clinical evidence that 2-PAM was effective. The only reported finding was a more rapid return of plasma pseudocholinesterase levels to normal in patients who received 2-PAM, as compared to those who did not. But in terms of long-term prognosis, this does not rule out the effectiveness of oxime therapy. Ideally, detailed studies are needed to evaluate the efficacy of 2-PAM, including for long-term prognoses. To date, however, there has been no sophisticated study of the Tokyo subway sarin attack in this vein.

One piece of evidence supporting the efficacy of 2-PAM to treat sarin toxicity has been the clinical benefit associated with it when treating toxicity due to organophosphorus pesticides. However, some experts now doubt whether such a benefit really exists. For example, Peter et al. (2006), using meta-analytic techniques, reevaluated the effects of oxime therapy in organophosphate poisoning. Not only did they find no beneficial effects, they reported possible adverse effects. The Cochrane Reviews for clinical evidence-based medicine (Buckley et al., 2005) reported no risk/benefit evidence supporting the use of oxime agents in organophosphate poisoning, but they did conclude that further detailed investigations are necessary.

According to reports about Iranian physicians who treated sarin toxicity during the Iran–Iraq war (Newmark, 2004), 2-PAM was not available on the front lines, and atropine alone was used for treatment. The doses of atropine used were considerably higher than those used in the Tokyo subway sarin attack, or that are generally recommended in the United States (Medical Letter, 2002). The Iranian protocol called for initial administration of 4 mg intravenously. If no atropine effects (improvement in dyspnea or decrease in airway secretions) were seen after 1–2 min, 5 mg was then administered intravenously over the next 5 min while the heart rate was monitored. A rise in heart rate of 20–30 beats per minute was diagnosed as an atropine effect. In severe cases, 20–200 mg was given. Regardless of the dose, the key to saving lives, according to this protocol, was how soon the atropine was administered.

Thus, treatment without the use of an oxime agent is possible. Of course, in countries where this is economically possible, treatment should use the combination of atropine, an oxime agent like 2-PAM, and diazepam. In addition, the use of autoinjectors for administration is also helpful. Unfortunately, terrorist attacks using sarin are also carried out in less economically developed countries; and even if the drugs are available, performance relative to cost needs to be considered. In this sense, preference should be given to the availability of atropine and diazepam. In other words, unless it is economically feasible, funds should be used to obtain atropine and diazepam rather than oxime agents, whose cost–benefit ratio is still inconclusive.

Based on data from 627 victims treated at St. Luke's Hospital (Okumura et al., 1998), the symptoms, listed in order of prevalence, were miosis (pupillary constriction; 90.5%), headache (50.4%), visual impairment (37.6%), eye pain (37.5%), dyspnea (29.2%), nausea (26.8%), cough (18.8%), throat pain (18.3%), and blurred vision (17.9%). Cases were categorized as severe if they involved seizures or respiratory arrest requiring mechanical ventilation, moderate for respiratory distress or fasciculations, and mild for eye symptoms only. Of 640 cases reported by St. Luke's Hospital, the degree of intoxication was severe in five victims, moderate in 107, and mild in 528, with nicotinic effects observed in those with moderate or severe symptoms.

In the Tokyo subway sarin attack, decontamination was not performed on site, and first-responders and healthcare workers initially did not wear PPE. As a result, of 1364 firefighting personnel, 9.9% became secondary victims. At that time, TV and newspapers reported on the sarin attack daily, and many people in Japan became scared of the sarin incident affecting them. At St. Luke's Hospital, 23% of hospital staff became secondary victims (Okumura et al., 1998). The percentage of secondary victims by hospital occupation was as follows: nursing assistants (39.3%), nurses (26.5%), volunteers (25.5%), doctors (21.8%), and clerks (18.2%). Thus, increased contact with a primary victim increased the risk of becoming a secondary victim. The percentage of secondary victims by hospital location were the chapel (45.8%), the intensive care unit (38.7%), the outpatient department (32.4%), the general ward (17.7%), and the emergency department (16.7%). The high rate of secondary victims in the chapel was attributed to poor ventilation and the large number of victims sheltered there. Because it was during winter, victims entered the chapel fully clothed. When they removed their coats, and every time they moved thereafter, some of the sarin trapped inside the clothing probably escaped, causing secondary exposure. Fortunately, none of the secondary victims died. However, if a higher concentration of sarin and more effective means of dispersion had been employed in the Tokyo attack, as had been done in the Matsumoto incident, then it is likely that some of the secondary victims would have died.

Within the context of risk communication, the so-called worried-well, who were concerned about having been exposed to the nerve agent, and those complaining of symptoms even though actual exposure was unlikely, also flocked to hospitals seeking treatment (Bloch et al., 2007). As previously mentioned, among patients treated at St. Luke's Hospital on the day of the attack, 90.5% had

miosis, an objective finding due to sarin exposure, but the remaining 9.5% were considered to be worried-well patients.

The reason or reasons for the small number of worried-well patients in the Tokyo subway sarin attack are unclear. Given the extensive coverage by the news media, who mentioned that victims were crowding into St. Luke's Hospital, persons without definitive symptoms, or those who were unsure whether they had been exposed but who did not want to add to the confusion, likely avoided going to that hospital, which created a kind of natural selection process. Another contributing factor may have been that the target of the attack was the government buildings in Kasumigaseki in the heart of Tokyo, which meant that many of the victims were probably well educated. Conversely, unfamiliarity with sarin and toxic gases in general may also have contributed to the low number of such patients. In either case, these observations should be reviewed from the perspective of risk communication.

4.2.3 Laboratory findings in sarin toxicity

According to inpatient records from St. Luke's Hospital, the most common laboratory finding related to sarin toxicity was a decrease in plasma cholinesterase (ChE) levels in 74% of patients. In patients with more severe toxicity, plasma ChE levels tended to be lower, but a more accurate indication of ChE inhibition is the measurement of erythrocyte ChE, as erythrocyte acetylcholinesterase (AChE) is considered "true ChE" and plasma ChE is "pseudo-ChE." However, erythrocyte ChE is not routinely measured, whereas plasma ChE is included in many clinical chemistry panels; thus, it can be used as a simple index for ChE activity. In both the Matsumoto and Tokyo subway sarin attacks, plasma ChE served as a useful index of sarin exposure. In 92% of hospitalized patients, plasma ChE levels returned to normal on the following day. In addition, inpatient records from St. Luke's Hospital showed elevated creatine phosphokinase and leukocytosis in 11% and 60% of patients, respectively. In severe cases such as the Matsumoto attack, hyperglycemia, ketonuria, and low serum triglycerides due to the toxic effects of sarin on the adrenal medulla were observed (Yanagisawa et al., 2006).

Acknowledgments

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Chapter 4.3

Part 3 Structural changes in the human brain related to sarin exposure

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The chemical sarin (*o*-isopropyl methylphosphonofluoridate) is a highly poisonous organophosphate (Lee, 2003). The main mechanism of acute sarin toxicity is irreversible inhibition of acetylcholinesterase. A single exposure to sarin causes chronic brain damage in animals, especially

in the hippocampus (Kadar et al., 1995; Chebabo et al., 1999; Abdel-Rahman et al., 2002); however, the long-lasting brain damage caused by a single exposure to sarin in human is poorly characterized. A number of studies have, however, explored a possible link between exposure to organophosphorus chemical weapons during the Gulf War and long-lasting medical problems, including changes in brain morphology (Haley et al., 1997, 2000; Couzin, 2004; Chao et al., 2010, 2011, 2014, 2015; Figueiredo et al., 2018). The compound was also deployed during the Iraq−Iran war and on Kurdish people in northern Iraq. In 2013, sarin was dropped in Damascus, killing more than 1400 Syrians including 426 children (Dolgin, 2013).

In the 1995 Tokyo subway sarin attack (TSSA), approximately 5500 civilians were exposed to sarin, 12 of whom died (Suzuki et al., 1995). The patients with acute sarin intoxication presented various symptoms, including muscle weakness and dyspnea. Almost all patients exhibited miosis and related symptoms, such as headache, blurred vision, ocular pain, and visual darkness, which are all typical symptoms of sarin intoxication (Lee, 2003; Ohbu et al., 1997). Approximately one-half of the patients exhibited abnormally low serum cholinesterase (ChE) levels a few hours after the exposure, which is also consistent with sarin exposure (Ohbu et al., 1997). However, longitudinal observations showed that various somatic complaints, ocular symptoms, and cognitive dysfunctions remained even 5 years after the sarin attack, despite ChE levels having returned to within the normal range (Kawana et al., 2001). The cause of these long-lasting medical problems has not been fully elucidated (Chen, 2012).

Yamasue and colleagues employed voxel-by-voxel whole-brain analyses of magnetic resonance images (MRI) to explore whether acute single-dose sarin intoxication in TSSA victims caused persistent morphological changes (Yamasue et al., 2007). The relationships between regional brain morphology, low serum ChE levels on the day of exposure, and current severity of somatic symptoms and cognitive dysfunctions were also explored in the TSSA victims to indicate a neurobiological basis of these long-lasting symptoms (Yamasue et al., 2007).

One hundred and ninety-one of the 582 TSSA victims, who were treated in the emergency room for acute sarin poisoning at St. Luke's International Hospital on March 20, 1995, had participated in a previous epidemiological study at the hospital in 2000 (Kawana et al., 2001). Of these 191 subjects, 38 right-handed victims agreed to participate in a neuroimaging study in 2000−2001, approximately 5−6 years after the TSSA. These subjects included 36 victims who participated in a study examining the effect of posttraumatic stress disorder (PTSD) on brain morphology (Yamasue et al., 2003). Nine of the 38 victims were diagnosed as having PTSD because of the attack. Seventy-six healthy volunteers matched for age, gender, handedness, and self and parental socioeconomic status also participated in the study.

Serum ChE levels, both on the day of the sarin attack and on the day of MRI scanning, were available for 22 victims. All of these 22 victims showed a decrease in serum ChE level on the day of the attack (117 ± 40, mean $\pm$ SD) compared with that on the day of the MRI scan (308 ± 62, $P < .001$). The percent reduction of serum ChE level on the day of the attack relative to that on the day of MRI scanning ranged from 42 to 81% (62 ± 12). The TSSA victims completed a self-reported questionnaire (Kawana et al., 2001), and of the 38 victims who participated in the neuroimaging study, 29 reported somatic complaints, 33 reported ocular symptoms, and 21 perceived forgetfulness, although memory function assessed with Wechsler Memory Scale-Revised (Wechsler, 1987) was observed to range from 100 to 119.

The 38 participants underwent MRI scanning and high-spatial resolution three-dimensional T1 images were acquired. Voxel-based morphometry (VBM) image processing, a fully automatic technique for computational analysis of differences in regional brain volume throughout the entire brain, was conducted. Statistical comparison between the TSSA and control groups was performed using an analysis of covariance model. To account for global anatomical variations, the intracranial volume was treated as a confounding covariate. Age and gender were also treated as confounding covariates because these measures can influence brain structure (Good et al., 2001a, 2001b). Small volume correction for multiple comparisons was used for regions that were predicted to be affected from animal studies of sarin intoxication, such as bilateral hippocampus (Kadar et al., 1995; Chebabo et al., 1999; Abdel-Rahman et al., 2002).

Although there was no group difference in total gray matter, total white matter, total CSF, or intracranial volume between the victims and the controls, the VBM revealed significantly reduced regional gray matter volume in the right insular and temporal cortex in the victims. A significant regional gray matter volume reduction in the left hippocampus was also detected with small volume correction. Moreover, the victims were also associated with significant regional white matter volume reduction in the left temporal stem close to the insular cortex (corrected $P < .05$). The regional white matter volumes in the right temporal stem and frontal lobe were also decreased in the victims, although the significance level was marginal ($.05 <$ corrected $P < .1$) (Fig. 4.3.1A).

To detect the effect of exposure to sarin, the subsequent analysis treated intracranial volume, age, and gender as confounding covariates, and the serum ChE level on the day of the attack and/or severity of clinical

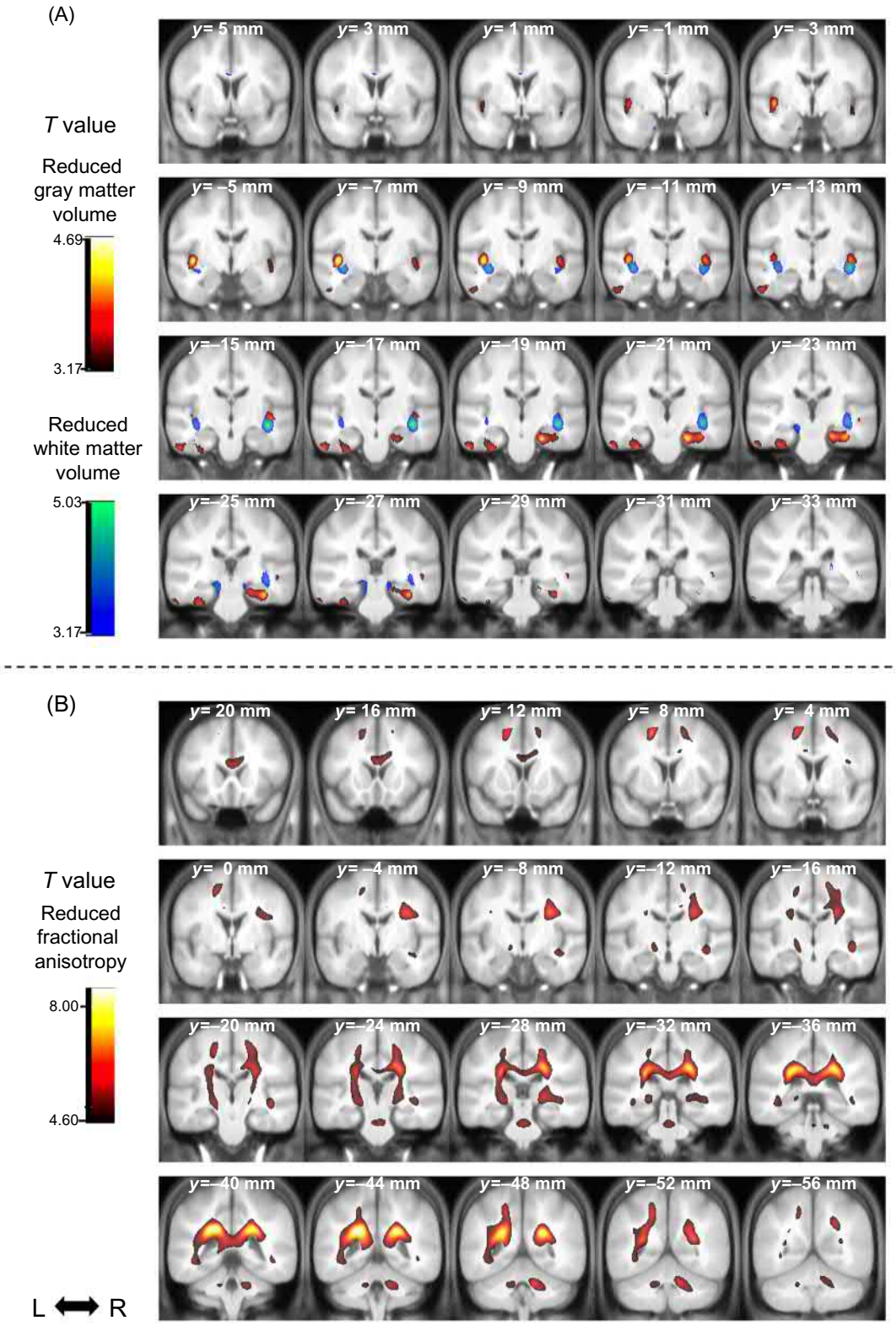

FIGURE 4.3.1 Regional brain morphology in sarin intoxication victims.
(A) Significant reductions in regional gray matter (*red-yellow*) and white matter (*blue-green*) volumes in the victims exposed to sarin compared with the controls are rendered onto the averaged image of the whole study sample ($n = 114$) (voxel threshold: uncorrected $P < .001$). The y-coordinate for each coronal slice in the Montreal Neurological Institute space is given in millimeters. (B) The regions showing a significant fractional anisotropy (FA) reduction in the victims exposed to sarin compared with the controls are rendered onto the averaged image of the whole sample ($n = 114$) (voxel threshold: corrected $P < .05$). The *y*-coordinate for each coronal slice in the Montreal Neurological Institute space is given in millimeters. *L*, left; *R*, right. *From Yamasue, H., Abe, O., Kasai, K., et al., 2007. Human brain structural change related to acute single exposure to sarin. Ann. Neurol. 61, 37–46.*

symptoms (somatic complaints, ocular problems, and forgetfulness) as the covariate of interest. An important difference between the victims of sarin intoxication and the control subjects, apart from the exposure to sarin, is a history of psychological stress. The region related to psychological stress was explored within the cluster, where the group comparison analysis showed regional volume reduction in victims compared with controls. A significant positive association with the serum ChE level at the time of the incident was found for the left subinsular white matter region, which was close to the temporal stem where the volume reduction was found in the group comparison (Fig. 4.3.2A). The serum ChE level at the time of the incident also showed positive correlations with the regional white matter volume in right frontal and left superior temporal regions with a trend toward significance ($.05 <$ corrected $P < .1$). There were no significant associations between regional gray matter volume and ChE indices. Furthermore, a significant negative correlation between the reduced regional white matter volume in the left temporal stem and the severity of the chronic somatic complaints was found in the victims (Fig. 4.3.2B). The severity of the somatic complaints was correlated with neither the severity of PTSD nor serum ChE level; therefore, the correlation between the somatic complaints and the left temporal stem cannot be attributed to these indirect effects. There were no other significant correlations between the severity of the ocular symptoms or forgetfulness and brain structure in the victims. The F-tests covering both ChE levels and somatic symptoms to address dependencies among these causal variables showed significant effects in the regional volume of the left subinsular white matter (corrected $P = .004$) and left temporal stem (corrected $P = .04$). The locations and statistical values of these effects are close to those of T-tests on the ChE levels but not on the somatic symptoms (subinsular: corrected $P = .007$; temporal stem: corrected $P = .002$); therefore, the correlations between the somatic symptoms

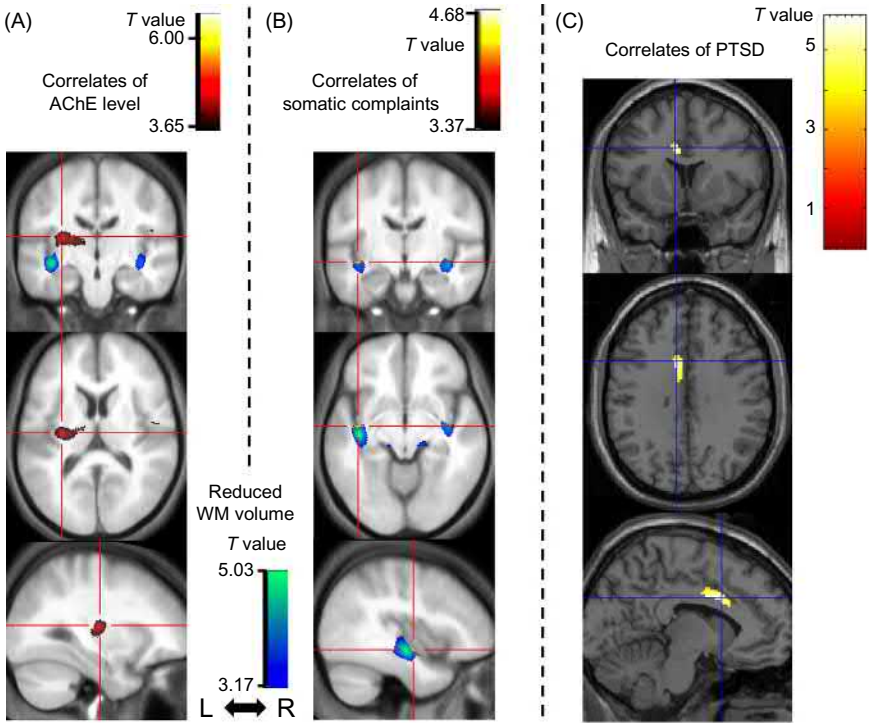

FIGURE 4.3.2 Neuroanatomical correlates of the decline in serum ChE level, somatic complaints, and posttraumatic stress disorder in sarin intoxication victims.

(A) The white matter (WM) region showing significant correlation with the effect of depressed cholinesterase (ChE) level was rendered onto the averaged image of the whole sample (*red-yellow*) ($n = 114$). (B) The white matter region showing a significant association with the somatic complaints was rendered onto the averaged image (*red-yellow*). The cluster, where the regional volume showed a significant association with the severity of somatic complaints, is included in the cluster, where victims had significantly smaller volumes than controls. (A, B) For comparison purposes, the regions showing significantly reduced regional white matter volume in the victims compared with the controls are overlaid and colored in blue (voxel threshold: uncorrected $P < .001$). (C) Regional differences between the victims with and without PTSD. Areas significantly reduced in the victims with PTSD compared with those without PTSD were confined to the anterior cingulate cortex and were rendered onto orthogonal slices of the normal template MR images. *L*, left; *R*, right. *From Yamasue, H., Kasai, K., Iwanami, A., et al., 2003. Voxel-based analysis of MRI reveals anterior cingulate gray-matter volume reduction in posttraumatic stress disorder due to terrorism. Proc. Natl. Acad. Sci. U.S.A. 100, 9039–9043; Yamasue, H., Abe, O., Kasai, K., et al., 2007. Human brain structural change related to acute single exposure to sarin. Ann. Neurol. 61, 37–46.*

and regional volume are independent from those between ChE levels and regional volume. In contrast, there were no brain voxels showing a significant correlation between an effect of psychological stress and the reduced regional brain volume in the victims within the regions showing group difference between the victims and controls, even when a liberal screening significance threshold of uncorrected $P < .001$ was utilized. Areas that were significantly reduced in the victims with PTSD compared with those without PTSD were confined to the anterior cingulate cortex (Fig. 4.3.2C) (Yamasue et al., 2003). Consequently, it can be concluded that the reduced regional volume in the right insular cortex, right temporal cortex, left hippocampus, and left temporal stem are related not to psychological stress but to the exposure to sarin.

All the subjects also underwent diffusion tensor imaging (DTI) in the same scanner and at the same time as 3D-T1. The voxel-by-voxel analysis of the fractional anisotropy (FA) maps created from DTI showed an extensive bilateral reduction in regional FA in the parietal and temporal lobes of the victims. Of note, the regional FA in the left temporal stem, where the VBM showed a significantly reduced white matter volume in the victims, was significantly reduced in the victims compared with controls. Furthermore, a regional FA in the brain stem, where a previous MR-spectroscopy study reported neurochemical abnormality in Gulf War veterans exposed to chemical nerve agent (Haley et al., 2000), was also significantly decreased in the victims (Fig. 4.3.1B). Furthermore, the decreased FA in the bilateral temporal stem and left subinsula significantly correlated with the more severe physical symptoms in the victims.

The crucial finding of the neuroimaging study by Yamasue et al. (2007) was a significant decrease in regional gray matter volume in the right insular and right temporal cortices, in the left hippocampus, and in regional white matter volume of the left temporal stem in the victims of sarin exposure compared with the matched controls not exposed to sarin. The regional volume of the left subinsular white matter showed a significant positive correlation with serum ChE levels at the time of the incident. Furthermore, reduced regional white matter volume of the left temporal stem was significantly related to the severe long-lasting somatic complaints in the victims. The DTI analysis showed that microstructural white matter integrity was significantly disrupted in widespread bilateral brain regions, including the parietal lobe, temporal stem, and brain stem, in the victims compared with the controls. It seems remarkable that the decreased FA was found in an area that also showed a significantly decreased white matter volume in the victims because these overlapping regional effects were inferred on the basis of independent data.

The victims included in the study demonstrated both a remarkable decrease in serum ChE levels and the presence of acute sarin intoxication symptoms. All of the victims were treated in the emergency room for acute sarin intoxication and showed a significant decrease in serum ChE levels; therefore, the severity of exposure to sarin was estimated to be at least intermediate (Brown and Brix, 1998). Most of the victims also suffered from various long-lasting somatic and cognitive symptoms, which is consistent with exposure to sarin at or above the intermediate level. Although most of the 38 victims suffered from various long-lasting symptoms, including somatic complaints, ocular symptoms, and forgetfulness, neuropsychological and occupational impairments were relatively mild.

The victims had significantly smaller regional volumes in the right insular cortex, right temporal cortex, left hippocampus, and left temporal stem than control subjects. Moreover, the regional volume in the left subinsular white matter, which was close to the left temporal stem where the significant volume reduction was found, showed a positive correlation with the serum ChE level at the time of sarin exposure. A limited number of previous studies have evaluated brain morphology of human subjects exposed to sarin, although animal studies have repeatedly reported dose-dependent (Abdel-Rahman et al., 2002) and progressive (Kadar et al., 1995) neuropathological change subsequent to sarin exposure (Chebabo et al., 1999). However, a limited number of studies using other research modalities have reported functional or chemical brain abnormalities in victims of sarin intoxication. Murata et al. (1997) reported significantly prolonged P300 auditory event-related potentials and P100 visual-evoked potential latencies in the TSSA victims. Matsuda et al. (1998) detected products of sarin hydrolysis in postmortem brain tissue of victims who died in the TSSA, although this analysis was limited to four individuals. Morphological changes in the brain have also been reported in Gulf War veterans. Haley et al. (2000) reported a reduced *N*-acetylaspartate-to-creatine (NAA/Cr) ratio in the putamen and brain stem of Gulf War veterans who had perceived chemical nerve agent exposure during wartime. Chao et al. (2010, 2011) reported a significant reduction in total gray and white matter volume in veterans with low-dose sarin and cyclosarin exposure (Chao et al., 2010, 2011). Significant reductions in the CA2 and CA3/dentate gyrus subfields of the hippocampus were reported in individuals exposed to low-dose chemical warfare agents (Chao et al., 2014), and remodeling of the white matter is suspected in the temporal stem, corona radiata, superior/inferior cingulum, internal and external capsule, inferior and superior fronto-occipital fasciculus, and nine superficial white matter areas located between the cortex and deep white matter (Chao et al., 2015).

Furthermore, animal studies show that ChE inhibitors have various delayed neurological and behavioral effects,

even at subtoxic doses (Scremin et al., 2003). The neuroimaging study that demonstrated regional brain volume reduction in victims of a single toxic sarin dose is partly consistent with these previous studies. Although overstimulation of muscarinic receptors and neuronal cell death induced by glutamate overflow subsequent to sarin exposure have been observed (Solberg and Belkin, 1997), the mechanism by which acute sarin exposure induces brain damage has yet to be clarified.

MRI examination (Yamasue et al., 2007) also showed a significant association between smaller regional white matter volume in the left temporal stem and more severe somatic complaints. Interestingly, the location of the neuroanatomical correlate of the somatic complaints was close to the insular cortex. Critchley et al. (2004) reported that activity and gray matter volume in the right insular cortex predicts individual variability in interoceptive awareness. Information concerning the internal state of the body is conveyed through a dedicated lamina-1 spinothalamocortical pathway that converges, with vagal afferents, to "interoceptive centers" in the insular cortex (Craig, 2002). The disruption of connections to the insular cortex thorough subcortical white matter fibers is linked to alterations in perceptions of internal bodily status (Manes et al., 1999). Furthermore, patients with insular stroke have more anergia, reduced activity, and tiredness than patients without insular lesions (Augustine, 1996). In summary, the insular cortex plays a crucial role in awareness of internal bodily status, such as pain, visceral, olfactory and gustatory sensations, and tiredness, especially in the emotional context (Augustine, 1996). Thus, the correlation found in this study indicates that regional volume reduction in this region related to sarin intoxication chronically influences subjective feeling states, such as tiredness, palpitations, and bodily pain among the victims.

The analysis utilizing DTI also revealed an FA reduction in a wider area than that showing volume reduction in the victims, in addition to associations between reduced FA and somatic complaints. DTI has been used to quantify the magnitude and directionality of tissue water mobility in three dimensions. Lower FA indicates less directionality and more random movement of water in all directions measured (Beaulieu, 2002). Previous studies have reported FA decreases without volumetric reduction in neuropsychiatric disorders (Kalus et al., 2005); therefore, FA may be more sensitive in finding morphological abnormalities than volumetric measurements. In addition to cerebral regions, the study by Yamasue et al. (2007) also found reduced FA in the brain stem. Haley et al. (2000) reported corroborating findings: Gulf War veterans exposed to organophosphorus chemical weapons and who exhibited mild cognitive and occupational impairment showed reduced *N*-acetylaspartate measured with 1H MR-spectroscopy, a marker of neural integrity.

The neuroimaging study (Yamasue et al., 2007) had limitations. First, the cross-sectional nature of the MRI examination and the absence of MRI scans and psychological assessments at the time of the incident cannot directly determine the etiology of the structural brain change. Second, the possible effects of psychological trauma on brain structure (MceEwen, 1999) cannot be totally ruled out, although a previous study indicated that smaller than normal hippocampal volume in patients with PTSD is likely to be a pre-existing vulnerability indicator rather than an acquired effect after traumatic events (Gilbertson et al., 2002). A bidirectional modulation of genes that regulate acetylcholine availability after stress and blockade of ChE has been observed (Kaufer et al., 1998), indicating possible synergy between the deleterious effects of ChE inhibitors and stress (Sapolsky, 1998). Third, a previous study reported the relationship between serum ChE levels and psychological stress in a sample overlapping that of the current study (Tochigi et al., 2002). Therefore, it is difficult to completely differentiate the effects of sarin intoxication from psychological stress. In this study, however, regional brain volume reduction showed a significant correlation with serum ChE levels but not with psychological symptoms, arguing against the possibility that psychological stress was a serious confound.

In conclusion, the MRI study of the TSSA victims (Yamasue et al., 2007) demonstrated in vivo evidence of a relationship between an acute single exposure to sarin and long-lasting morphological changes in specific brain regions. Furthermore, the findings also indicate that the increased sensitivity to internal bodily status in the victims is related to morphological brain damage, including in the insular and neighboring subcortical white matter: the "interoceptive centers." Therefore, these findings shed light on the neural background of unexplained somatic problems.

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Chapter 5

Early and delayed effects of sulfur mustard in Iranian veterans after the Iraq–Iran conflict

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5.1 Introduction

5.1.1 Brief chemistry

Sulfur mustard (SM), which is bis(2-chloroethyl) sulfide and is also known as mustard gas, was first synthesized in 1822 by Despretz. SM is an oily liquid that is colorless if pure, but it normally ranges from pale yellow to dark brown. Iranian veterans have described it as having a slight garlic or horseradish odor. It has a density of 1.27 g/mL, a melting point of 14.4°C, and a boiling point of 217°C. SM is only 0.05% soluble in water (Kikilo et al., 2008; Pechura and Rall, 1993).

SM is generally regarded as a "persistent" chemical agent because of its low volatility. In cool weather there is little vapor; however, mustard's evaporation increases as the temperature increases. At higher temperatures, such as those in the Middle East during the hot season (38°C–49°C), mustard vapor becomes a major hazard (Pechura and Rall, 1993; Sidell et al., 1997; Kikilo et al., 2008).

5.1.2 Summarized historical uses

SM has been the most widely used chemical warfare agent (CWA) of the past century. It was first used extensively in World War I (WWI) between 1914 and 1918. Despite the Geneva Protocol in 1925 on the prohibition of CWA, SM was used by Italian troops in Ethiopia (1935–36) and by Egyptian forces in Yemen (1963–67). The greatest military use of SM was by the Iraqi Army against the Iranian soldiers and even civilians in Sardasht and Halabjah between 1983 and 1988, resulting in more than 100,000 chemical casualties (Balali-Mood and Hefazi, 2005; Salem et al., 2008).

5.2 Types and routes of exposure

Based on the situation involved, several types of exposure including single, multiple, secondary, subclinical, and chronic may occur. Most human cases of SM poisoning have occurred during armed conflicts and most accidents involved a single exposure (Pechura and Rall, 1993; Sidell et al., 1997; Balali-Mood and Hefazi, 2005; Kikilo et al., 2008; Salem et al., 2008). Multiple low SM exposures occurred occupationally, during WWI and in the Iran–Iraq conflict (Balali-Mood and Hefazi, 2005; Salem et al., 2008). First aid workers and nursing and medical staff who cared for SM casualties in the field clinics and hospitals during the Iraq–Iran War without proper personal physical protection have become intoxicated. Some of them (between 5% and 25%) are now suffering from the delayed toxic effects of SM and have disabilities (Balali-Mood and Hefazi, 2005).

Low-level SM exposure with or without symptoms but with delayed or long-term health effects has been described in detail (Mandel and Gibson, 1917; Hurst et al., 2001; Balali-Mood et al., 2005a,b; Balali-Mood et al., 1986a). Subclinical exposure to SM in some Iranian combatants induced delayed toxic effects (Ghanei et al., 2004a,b,c). Chronic SM exposure is usually occupational. Some factory workers in Japan and in the United Kingdom were reported to have had SM poisoning and even malignancies attributable to SM (Brown, 1949; Nishimoto et al., 1970).

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Inhalation is the major route of exposure that induces respiratory and systemic toxicity after absorption across the lung surface (Sidell et al., 1997; Balali-Mood and Hefazi, 2005; Hefazi et al., 2005). However, SM is a vesicant or blistering agent that has direct toxic effects on the skin, producing erythema, blistering, epidermolysis, and necrosis. It is a lipid-soluble compound and thus can be readily absorbed across the skin (Balali-Mood and Hefazi, 2005; Hefazi et al., 2006).

The eyes are the organs most sensitive to SM. This marked susceptibility is attributed to ocular features, including the aqueous−mucous surface of the cornea and conjunctiva as well as the high turnover rate and intense metabolic activity of the corneal epithelial cells (Pickard, 1919; Etezad-Razavi et al., 2006).

SM may also enter the body via oral ingestion. Some Iranian combatants were observed during the Iran−Iraq War who had ingested food contaminated by SM and subsequently became intoxicated. They experienced nausea, vomiting, hematemesis, abdominal pain, and dyspnea. SM may also be absorbed through the lower gastrointestinal (GI) tract (Balali-Mood and Hefazi, 2005). Injection is a very rare route of SM intoxication and has not been reported in humans.

5.3 Human toxicity

Exposure to very high doses of SM in the field may induce convulsions and death in less than 1 h (World Health Organization, 1970; Balali-Mood et al., 1986a; Sidell et al., 1997; Balali-Mood and Hefazi, 2005). Such observations have not been reported during the Iran−Iraq War. Acute toxic effects generally appear after variable periods of latency depending on the dose, mode of exposure, the environmental temperature, and the individual (Anslow et al., 1946; World Health Organization, 1970; Balali-Mood et al., 1986; Pechura and Rall, 1993; Balali-Mood and Hefazi, 2005).

Subacute exposure occurred during the Iran−Iraq War and in the workers in SM munitions factories. However, this type of exposure may present as mild acute SM intoxication, as a complication in the respiratory tract, or even as malignancy (Balali-Mood, 1986; Easton et al., 1988).

Delayed toxic effects of SM have been documented. The first report of delayed toxic effects in Iranian veterans was reported in 1986 (Balali-Mood, 1986). Several articles on the delayed toxic effects and complications of SM in Iranian veterans have since been published (World Health Organization, 1970; Balali-Mood, 1986; Ghanei et al., 2004a,b,c; Balali-Mood et al., 2005a,b; Hefazi et al., 2005; Etezad-Razavi et al., 2006; Hefazi et al., 2006).

The workers who were chemically exposed to mustard agents in British and Japanese munitions factories developed chronic respiratory effects, including chronic bronchitis, bronchiectasis with progressive emphysema, and narrow attenuated bronchioles (Brown, 1949; Nishimoto et al., 1970; Easton et al., 1988).

5.4 Main mechanisms of toxicity

The monofunctional mustards have one alkylating site and, therefore, can attack and break the DNA at specific nucleotides. Although SM reacts with RNA, proteins, and phospholipids, the general consensus is that a DNA alkylate plays an important role in delayed toxic effects (Walker, 1971; Ball and Robert, 1972). The major alkylating site of nucleic acids of mammalian origin is the nitrogen residue of guanine (Wheeler, 1962). Cell death from DNA cross-linking is delayed until the cell replicates its DNA or undergoes division. At higher cellular exposures, however, mechanisms other than DNA cross-linking become important and produce more rapid cell death. The acute damage to the cornea, mucous membranes, and skin seen after SM exposure is probably generated by one or more of these other mechanisms. One mechanism that may be involved in acute damage is nicotinamide adenine dinucleotide depletion. Other potential mechanisms of cell death are related to rapid inactivation of sulfhydryl containing proteins and peptides, such as glutathione. These sulfhydryl compounds are critical in maintaining the appropriate oxidation reduction state of cellular components. Glutathione is also thought to be critical in reducing reactive oxygen species in the cell and preventing peroxidation and loss of membrane integrity (Rankin et al., 1980; Eklow et al., 2004). Tumor necrosis factor-α is involved in SM-induced skin lesions (Worsmer et al., 2005).

5.5 Target organs and acute clinical features

Acute toxic effects of SM are mostly observed in the eyes, respiratory tract, and skin. These organs are thus known as the target organs, because their involvement is more prominent here than in the other organs. However, neuropsychiatric symptoms and signs and digestive and cardiovascular dysfunctions were also observed. Frequencies of clinical features of acute SM intoxications in 233 Iranian patients treated in Mashhad, Iran, are shown in Fig. 5.1.

Eyes are the organs that are most sensitive to SM. The first symptoms of SM exposure usually occur in the eyes. The Iranian patients with SM exposure reported itching and burning sensation of the eyes, leading to acute conjunctivitis, blepharospasm, and even keratitis. In the acute stage, the limbal region frequently presents a marbled appearance in which porcelain-like areas of ischemia are

surrounded by blood vessels of irregular diameter. Later, the vascularized scars of the cornea often contain deposits of cholesterol, calcium, and fat (Giraud, 1917; World Health Organization, 1970; Balali-Mood et al., 1986a,b; Balali-Mood et al., 1986; Balali-Mood and Hefazi, 2005).

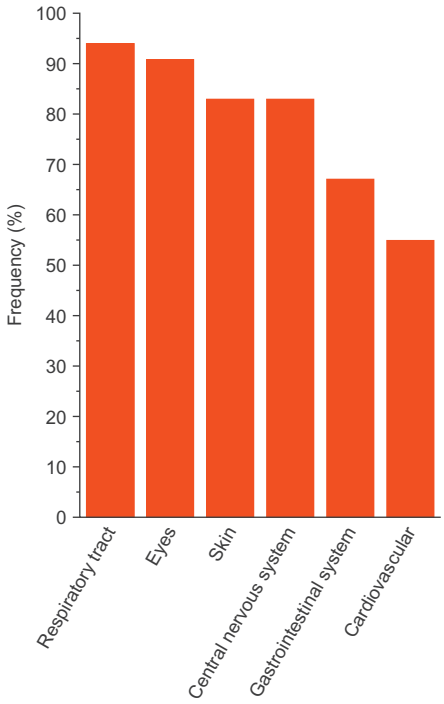

FIGURE 5.1 Frequency of clinical features of acute SM poisoning in different organs of 233 Iranian patients treated in Mashhad, Iran, a few days after exposure.

Respiratory effects occur in a dose-dependent manner from the nasal mucosa to the terminal bronchioles (World Health Organization, 1970; Balali-Mood et al., 1986a,b). Acute pulmonary effects commence with a tracheobronchitis, followed by bronchopneumonia, adult respiratory distress syndrome, and even pulmonary emboli in severely intoxicated patients, which may lead to death, mostly during the second week after SM exposure. Chest X-ray (CXR) of the Iranian SM-intoxicated patients revealed fewer abnormalities than the clinical manifestations. However, the severely intoxicated patients showed abnormal CXR results. CXRs of an Iranian patient with bronchopneumonia caused by acute SM poisoning before and after treatment in 1985 are shown in Fig. 5.2.

Direct toxic effects of SM on the skin are the main apparent effects that lead to it being called a vesicant or a blistering agent. Erythema and blisters are the most common skin manifestations caused by SM exposure. A soldier who had a separate suit and face mask suffered from skin erythema and blisters on his neck 3 days after SM exposure, as shown in Fig. 5.3.

A German and an Iranian medical toxicologist (first author) classified the cutaneous mustard gas lesions as described under the clinical manifestations (Helm and Balali-Mood, 1988).

GI effects after SM exposure have been documented in some studies. Destruction of the mucosa and shedding of the epithelial elements, however, begin days after exposure, resulting in the loss of large volumes of fluid and electrolytes (Papirmeister et al., 1991). Acute gastroduodenitis with hemorrhagic erosions, acute desquamative enteritis, and severe hemorrhagic necrotic colitis were

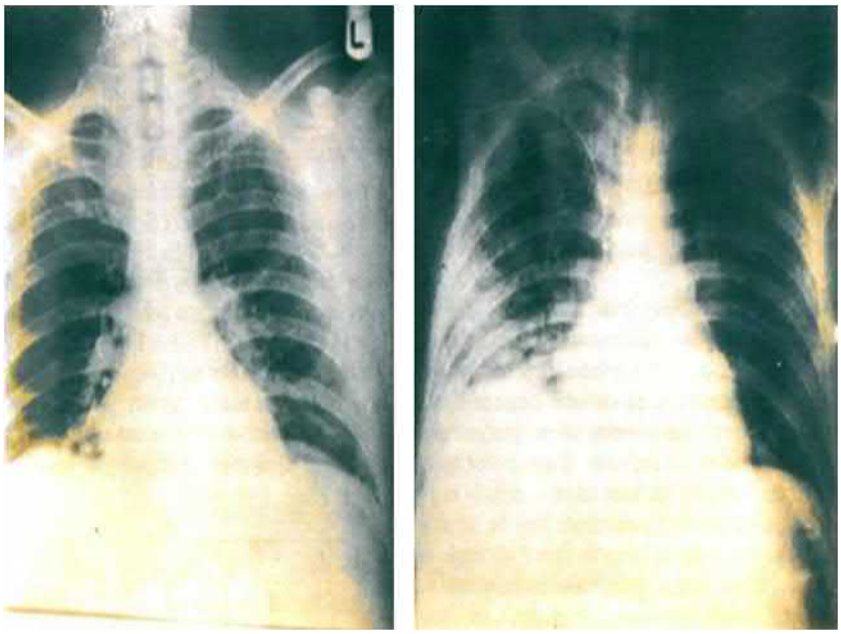

FIGURE 5.2 Chest X-rays of an Iranian patient with bronchopneumonia due to acute SM poisoning before (right) and after (left) treatment 8 and 19 days after exposure in 1985.

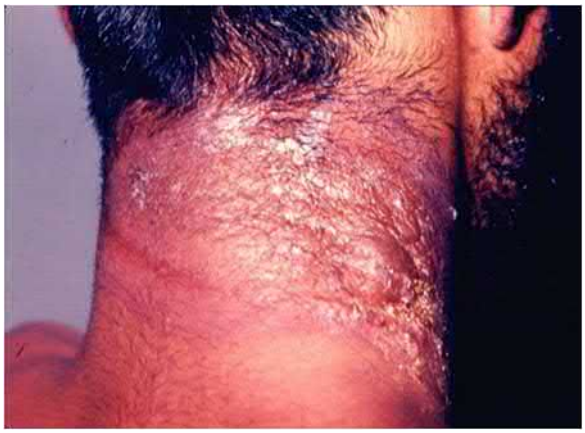

FIGURE 5.3 A soldier who had a separate suit and face mask revealed skin erythema and blisters on his neck 3 days after SM exposure in 1985.

reported in WWI veterans (Canelli, 1918) but were not observed in the Iranian veterans.

Extremely heavy exposure to SM can cause central nervous system (CNS) excitation, leading to convulsions in animals (Anslow et al., 1946). Balali-Mood et al. (1986a,b) reported convulsions in six Iranian veterans who were hospitalized during the early stages of their intoxication (Balali-Mood et al., 1986a,b). Most casualties from WWI and from the Iran–Iraq conflict, however, revealed mild and very nonspecific neurological effects, such as headache, anxiety, restlessness, confusion, and lethargy (Canelli, 1918; Balali-Mood et al., 1986a,b). A frequent long-term complication in patients exposed to SM is delayed neuropathic toxicity, which was underrepresented in most previous studies (Thomsen et al., 1988).

5.6 Hematoimmunological complications

SM, as an alkylating agent, is particularly toxic to rapidly proliferating cells such as lymphoid and bone marrow cells. Leukocytosis is common within the first few days after exposure. White blood cell (WBC) counts then begin to decrease starting from the third or fourth day after exposure and reach their minimum level at approximately the ninth day. This leukopenia is followed by a decrease in megakaryocytes and, finally, in the erythropoietic series (Willems, 1989; Tabarestani et al., 1990; Balali-Mood et al., 1991). Bone marrow biopsies have shown hypocellular marrow and atrophy involving all elements (Tabarestani et al., 1990). If cytopenia is not marked and there are still remaining stem cells, then recovery will occur as the patient recovers (Krumbhaar and Krumbhaar, 1919; Tabarestani et al., 1988; Tabarestani et al., 1990; Balali-Mood et al., 1991; Mahmoudi et al., 2005). The bone marrow studies reveal a severe decrease in cellularity and fat replacement and also nuclear changes, such as budding, binuclear, and karyorrhexis in erythrocyte precursors. The toxic effects of SM on the hematopoietic system are dose-dependent, and it is concluded that SM causes aplastic or ineffective hematopoiesis (Tabarestani et al., 1988). Severe leukopenia, however, is an ominous sign, leading to secondary infections and higher mortality rates in these patients. SM victims with WBC counts of 200 cells/mL or less died during their initial admissions (Willems, 1989).

SM poisoning could result in the impairment of both humoral and cellular immune functions (Dayhimi et al., 1988; Zandieh et al., 1990; Ghotbi and Hassan, 2002). Along with the appearance of clinical disorders, both C3 and C4 titers showed an increase, followed by a gradual decrease, over 1 year. The majority of SM-exposed patients had increased levels of IgG and IgM during the first weeks and up to 6 months after exposure (Ghotbi and Hassan, 2002).

A decrease of cell-mediated immunity has been observed in Iranian veterans 1, 2, and 3 years after exposure (Zandieh et al., 1990). Natural killer (NK) cells, which are known to be one of the most important components of cellular immunity, have been found to be significantly lower in patients with severe respiratory complications 10 years after exposure (Ghotbi and Hassan, 2002).

Hematological and immunological complications of 40 patients 16–20 years after severe SM intoxication in comparison with 35 controls were reported (Balali-Mood et al., 2005a,b; Mahmoudi et al., 2005). Total WBC and red blood cell (RBC) counts and hematocrit (HCT) levels were significantly higher in the patients than in the control group. The percentages of monocytes and $CD3^+$ lymphocytes were significantly higher, and the percentage of CD16 + 56 positive lymphocytes (NK cells) was significantly lower in patients than in the control group. Other hematological and flow-cytometric parameters did not show any significant difference between the two groups. Serum IgM and C3 levels were significantly higher in the patients in comparison with the controls. Other immunoglobulins and complement factors did not show any significant difference between the two groups, as shown in Table 5.1.

5.7 Delayed clinical complications

The main target of the long-term complication of SM poisoning is the respiratory system. In a study of incidence of common late complications of SM poisoning in lungs, eyes, and skin in 34,000 Iranians, the lungs were found to be the most affected organ (Khateri et al., 2003). In another study, the most common clinical complications of SM in different organs of 40 Iranian veterans 16–20 years after exposure showed that the respiratory tract was

TABLE 5.1 Hematological and immunological changes in 40 patients, 16–20 years after severe SM intoxication comparing with 35 healthy subjects.

Parameters	Patient mean SD	Control mean SD	*P*-Value
WBC (1000/mm^3)	7.24 ± 1.90	5.79 ± 1.12	.025
RBC (million/mm^3)	5.46 ± 0.45	5.19 ± 0.28	.035
Hb (mg/dL)	15.9 ± 0.7	15.6 ± 0.7	.223
HCT (%)	48.3 ± 3.5	45.5 ± 1.9	.047
PLT (1000/mL)	255 ± 99	238 ± 10.1	.594
Lymphocyte (%)	31.5 ± 8.4	30.5 ± 10.8	.651
Monocyte (%)	4.8 ± 1.6	3.9 ± 1.1	.013
Polynuclear (%)	63.8 ± 8.7	65.4 ± 8.7	.327
IgA (mg/dL)	302.6 ± 142.1	233.1 ± 59.3	.154
IgM (mg/dL)	235.3 ± 84.8	136.8 ± 58.3	.0001
IgG (mg/dL)	1438.6 ± 485.1	1140.0 ± 244.2	.065
IgE (IU)	92.4 ± 112.1	86.5 ± 164.3	.161
C3 (micg/dL)	109.8 ± 30.1	90.9 ± 14.8	.030
CD3 (%)	71.1 ± 8.6	65.6 ± 10.7	.037
CD16 + 5 (NK cells %)	11.6 ± 5.8	17.5 ± 9.6	.006

TABLE 5.2 Frequency of delayed complications of SM in different organs of 40 Iranian veterans in Mashhad 16–20 years after exposure.

Organs	Number of patients	Percentages
Respiratory tract	38	95
Peripheral neuromuscular	30	75
Skin	29	72.5
Eyes	27	67.5

involved in 95% of the patients, as shown in Table 5.2 (Balali-Mood et al., 2005a,b).

5.8 Respiratory tract

Respiratory complications are the greatest cause of long-term disability among people with SM exposure. A pulmonologist who investigated the pulmonary complications in Iranian veterans in 2007 named it mustard lung (Ghanei and Adibi, 2007).

A triad of cough, expectoration, and dyspnea has been found to be present in more than 80% of Iranian veterans 3 years after their initial exposure (Balali-Mood, 1992; Balali-Mood et al., 1986a). Hemoptysis (mainly streaky), chest tightness, chest pain, and nocturnal dyspnea are also frequent. The main objective clinical findings are generalized wheezing (the most common sign), crackles, decreased lung sounds, clubbing, and cyanosis (Balali-Mood, 1992; Balali-Mood et al., 1986a,b; Ghanei and Adibi, 2007).

Pulmonary function testing has revealed more obstructive patterns than restriction, and approximately half of these obstructive spirometric results are reversible in response to inhaled bronchodilators. FVC, FEV1, and FEV1/FVC (FEV1%) have all been found to be significantly lower in SM-intoxicated veterans in comparison with healthy nonexposed subjects and CWA survivors who had used a gas mask at the time of attack (Balali-Mood, 1992; Balali-Mood and Hefazi, 2005; Balali-Mood et al., 2005a,b; Ghanei and Adibi, 2007).

Abnormal spirometric findings in general and restrictive patterns in particular tend to increase over time (Balali-Mood and Hefazi, 2005; Balali-Mood et al., 2005a,b; Ghanei and Adibi, 2007). A study of 77 subjects who were present in a contaminated area and had no acute signs and symptoms at the time of exposure, but who now have respiratory disorders, indicates that subclinical exposure to SM can be responsible for the occurrence of delayed respiratory complications such as bronchiectasis and bronchiolitis obliterans (Ghanei et al., 2004a,b,c).

CXR findings in patients with late respiratory complications of SM have been described as increased bronchovascular markings, hyperinflation, bronchiectasis, pneumonic infiltration, and radiologic evidence of pulmonary hypertension (Balali-Mood et al., 2005a,b; Ghanei and Adibi, 2007). However, CXR is not sensitive enough for the detection of respiratory complications in these patients and high-resolution computed tomography of the chest may be required as the diagnostic imaging procedure of choice (Bagheri et al., 2003; Balali-Mood et al., 2005a,b; Bakhtavar et al., 2008). A study of 197 Iranian veterans 10 years after a single heavy exposure to SM revealed the development of a series of delayed destructive pulmonary sequelae, such as chronic bronchitis (58%), asthma (10%), bronchiectasis (8%), large airway narrowing (9%), and pulmonary fibrosis (12%). Each of these complications is described in more detail (Emad and Rezaian, 1997).

5.8.1 Chronic bronchitis

Several studies have reported chronic bronchitis as the most common late complication of the respiratory system resulting from war exposure to mustard gas (Balali-Mood, 1992; Emad and Rezaian, 1997; Ghanei et al., 2004a,b,c, 2005, 2006a,b; Balali-Mood et al., 2005a,b). Hypoxemia and hypercapnea are commonly observed in moderate to severe cases, leading to cor pulmonale and respiratory failure in the final stages of the disease (Emad and Rezaian, 1997; Balali-Mood and Hefazi, 2005; Balali-Mood et al., 2005a,b). Infection of the respiratory tract, resulting in bronchopneumonia, is also a common problem that is often complicated by septicemia (Balali-Mood et al., 1986a,b, 2005a,b; Balali-Mood and Hefazi, 2005).

5.8.2 Asthma

Airway hypersensitivity, manifested as typical attacks of breathlessness, wheezing, and nocturnal cough, as well as a reversible obstructive pattern on pulmonary function tests, have been reported between 4 weeks and 20 years after SM inhalation. Patients with chronic bronchitis may also have some degree of bronchospasm, which does not respond to bronchodilators. Attacks of bronchospasm are characteristically triggered by respiratory infections, environmental allergens, and cold weather (Emad and Rezaian, 1997; Bijani and Moghadamnia, 2002; Ghanei et al., 2004a,b,c, 2005, 2006a,b; World Health Organization and Perry-Robinson, 2004; Balali-Mood et al., 2005a,b). New techniques, such as impulse oscillometry (IOS), have been used for evaluation of airway dysfunction. However, it was found to be less sensitive than spirometry in spotting small airways obstructions. IOS is a good diagnostic method in the detection of pulmonary involvement in uncooperative patients (Ghanei et al., 2004a,b,c).

5.8.3 Bronchiectasis

Direct effects of SM on the bronchial wall mucosa and more recurrent respiratory infections after inhalation of SM are known to be responsible for the development of bronchiectasis. Both the severity and frequency of bronchiectatic lesions tend to increase over the long-term, as evidenced by a study of 40 Iranian veterans with severe late complications of SM poisoning. These lesions usually begin bilaterally in the lower lobes and then progress toward the middle lobe and the lingula.

In severe cases with extensive bronchiectatic lesions, pulmonary hypertension and, ultimately, cor pulmonale may occur (Sohrabpour, 1992; Hosseini, 1998; Aslani and Cheraghali, 2000; Ghanei et al., 2004a,b,c; Balali-Mood et al., 2005a,b).

5.8.4 Large airway narrowing

Airway narrowing, attributable to scarring or granulation tissue, is late sequelae of acute injuries to the trachea and large bronchi, usually developing 2 years after exposure (Aslani and Cheraghali, 2000; Sohrabpour, 1992; Ghanei et al., 2004a,b,c). A study of 19 Iranian veterans with large airway narrowing caused by SM revealed stenosis in the trachea (seven cases), main bronchi (eight cases), and lobar bronchi (four cases) (Ghanei et al., 2004a,b,c). In contrast to stenosis caused by prolonged intubations, there is no predilection in the right main bronchus (Balali-Mood et al., 2005a,b). The major problem in these patients is the recurrence of the lesion, which usually occurs 6 months after treatment (Aslani and Cheraghali, 2000).

5.8.5 Pulmonary fibrosis

Late-onset pulmonary fibrosis has been reported in several Iranian veterans with combat exposure to SM (Aslani and Cheraghali, 2000; Balali-Mood et al., 2005a,b). The analysis of bronchoalveolar lavage fluid from patients with mustard gas inhalation showed that these patients

have an ongoing local inflammatory process of the lower respiratory tract, resulting in the development of pulmonary fibrosis years after the initial exposure. Histopathological examination of transbronchial lung biopsy (TBLB) samples of SM-exposed veterans revealed variegated fibrosis, diffuse fibrosis, and an absence of fibrosis in 86%, 4%, and 10% of the patients, respectively. Usual interstitial pneumonitis accounted for 97% of all cases of fibrosis (Emad and Rezaian, 1997). In another study, electron microscopic examination of seven TBLB specimens was performed in a WHO research center in Japan. Abnormal findings included: (1) proliferation, desquamation, and degeneration of the bronchial epithelial cells; (2) interstitial fibrosis or fibrosing alveolitis; and (3) increased type I and type II alveolar epithelial cells as well as hyperplasia of ciliated and goblet cells (Sohrabpour, 1992). Inflammation and fibrotic processes in the lung tissue of SM-exposed patients may be progressive (Ghanei et al., 2004a,b,c). Diffusing lung capacity could be used as an objective monitor of the degree of fibrosis and also as a good predictor for prognosis (Emad and Rezaian, 1997).

5.9 Peripheral neuromuscular complications

Electromyography (EMG) and nerve conduction velocity (NCV) of 40 Iranian veterans with severe late manifestations of SM poisoning revealed abnormalities in the peripheral nervous system of 77.5% of the patients. NCV disturbances were more common in sensory nerves compared with motor nerves and were more prevalent in the lower extremities than in the upper extremities. EMG recordings revealed a normal pattern in 24 (60%) patients, incomplete interference with normal amplitude in 6 (15%) patients, and incomplete interference with low amplitude in 10 (25%) patients. NCV and EMG disturbances in both upper and lower extremities were mostly symmetric (Ghanei et al., 2004a,b,c; Balali-Mood et al., 2005a,b).

5.10 Dermal delayed effects

The occurrence and persistence of lesions after SM exposure are directly related to the duration and severity of exposure. Injury that results in erythema and edema without vesicle formation is almost always followed by complete healing and nonresidual effects (Chiesman, 1944; Balali-Mood and Hefazi, 2005). Blistering and necrotic wounds, however, cause permanent residual effects. The first report of delayed toxic effects of SM poisoning in 236 Iranian veterans 2 years after exposure revealed late skin effects such as hyperpigmentation (34%), hypopigmentation (16%), and dermal scarring (8%) (Balali-Mood et al., 1986a,b). The most common skin symptom among these patients was itching, followed by a burning sensation and desquamation. These symptoms are basically caused by dryness of the skin and become worse in dry weather and after physical activity. A more recent study of 40 Iranian veterans who were heavily exposed to the gas 16–20 years previously revealed the most common cutaneous lesions to be hyperpigmentation, erythematous papular rash, dry skin, multiple cherry angiomas, atrophy, hypopigmentation, and hypertrophy. These lesions were found on the genital areas (48%), the back (48%), the front thorax and abdomen (44%), lower extremities (mainly inguinal) (44%), upper extremities (mainly auxiliary) (41%), and the head and neck (15%). Dry skin was more prominent in the extremities. Hyperpigmentation in some patients had the appearance of pigmented xerodermoid, which is a diffuse hyperpigmented area with superimposed macular hypopigmentations and hyperpigmentations (Balali-Mood et al., 2005a,b; Hefazi, et al., 2006).

In another study, the cutaneous lesions of 500 SM-exposed Iranian veterans were compared with 500 unexposed veterans. An association was found between SM exposure and late skin lesions such as severe dry skin, hyperpigmentation and hypopigmentation, localized hair loss, eczema, and chronic urticaria. There was also a higher incidence of vitiligo, psoriasis, and discoid lupus erythematosus among SM-poisoned patients. This could be attributable to the immunological basis of these disorders and to the fact that SM has adverse long-term effects on the immune system. Previously injured sites have been reported to be sensitive to subsequent mechanical stimulation and showed recurrent blistering after mild injury (Fekri and Janghorbani, 1992).

Histopathological examination of skin biopsy samples has revealed nonspecific findings including epidermal atrophy, keratosis, and basal membrane hyperpigmentation. Nonspecific fibrosis and melanophages have also been observed within the dermis (Fekri and Janghorbani, 1992; Balali-Mood et al., 2005a,b; Hefazi et al., 2006). Occupational exposure to SM has been demonstrated to cause a variety of skin changes, including pigmentary disorders, skin ulcers, and cutaneous cancers (Khehr, 1984).

5.11 Ophthalmologic complications

In less than 1% of patients with battlefield exposure to SM, a delayed type of ulcerative keratopathy may develop, leading to late-onset blindness (Hughes, 1942; Blodi, 1971; English and Benett, 1990; Pleyer et al., 1999; Javadi et al., 2000). The maximum delayed toxic effects usually occur 15–20 years after initial exposure, although latency periods as long as 40 years or as short as 6 years have also been reported (Solberg et al., 1997;

Javadi et al., 2000; Etezad-Razavi et al., 2006). Patients are usually symptom-free for an indefinite number of years when delayed keratitis develops, characterized by photophobia, lacrimation, and failing vision (Pleyer et al., 1999). Vascularized scars of the cornea are covered with crystal and cholesterol deposits, leading to a worsening of the opacification, recurrent ulcerations, and sometimes corneal perforation. Opacification of the cornea is seen predominantly in the lower and central portions, whereas the upper part is often protected by the eyelid (Solberg et al., 1997; Pleyer et al., 1999). Surprisingly, lesions even recur after corneal transplantation (Javadi et al., 2000). The exact pathogenesis of this condition is unknown, but degenerative processes and immune reactions against corneal proteins (collagen−mustard compound) have been suggested as the cause of long-term damage (Solberg et al., 1997). Unfortunately, there has been no report of any long-term studies of mustard gas workers to determine their ocular status after prolonged occupational exposure.

5.12 Psychiatric complications

Casualties from WWI and from the Iran−Iraq conflict were noted to be long-term mood and anxiety disorders, as well as posttraumatic stress disorder (Balali-Mood, 1986a,b; Tabatabaee, 1988; Hashemian et al., 2006). Debility, loss of vitality, impaired concentration, sensory hypersensitivity, diminished libido, weakened potency, neuralgic symptoms, and disorders in autonomic regulation are common manifestations. Neuropsychiatric evaluation of 1,428 Iranian veterans 3−9 years after exposure to SM revealed anxiety (15%), depression (46%), personality disorders (31%), convulsions (6%), and psychosis (3%) (Tabarestani et al., 1988). Disorders of consciousness (27%), attention (54%), emotion (98%), behavior (80%), thought process (14%), and memory (80%) were studied in 70 patients 3−5 years after SM exposure (Balali-Mood, 1986a,b). Depression and posttraumatic stress in Iranian survivors of chemical warfare, mostly SM exposure, were also reported (Javadi et al., 2000). In another study, decreased libido and impotence were recorded in 52% and 9% of patients, respectively. Quite interestingly, 10% of the patients revealed an increased libido. Functional photophobia and aphonia and effort syndrome have also been reported (Balali-Mood et al., 2005a,b).

5.13 Carcinogenicity

SM is genotoxic because of its reactions with DNA, which is an important first step in carcinogenesis. Although most cells possess effective DNA repair mechanisms, these are not always effective in the case of SM damage. Alkylation of *O*6-guanine by SM seems to be critical. *O*6-ethylthioethylguanine is a poor substrate for the DNA repair enzyme *O*6-alkylguanine-DNA alkyltransferase (Ludlum et al., 1986). Therefore, this *O*6 lesion may be the most important mutagenic lesion. However, only limited data are available regarding the specific mutations produced by SM. Mutations in a tumor suppressor or an oncogene gene can favor a proliferate advantage of a clonal cell. Notably, alterations in the p53 tumor suppressor gene have been described in Japanese mustard gas workers (Takeshima et al., 1994). However, most of the lesions in this population were similar to smoking-related mutations. Mutations in lymphocytes at the hypoxanthine phosphoribosyltransferase (hprt) gene locus have also been reported (Yanagida et al., 1988).

5.14 Reproductive complications

The effects of SM exposure during pregnancy are unknown. Data addressing the productive toxicities of SM in human models are both lacking and contradictory (Azizi et al., 1995).

5.15 Cardiovascular complications

Cardiovascular complications such as myocardial perfusion abnormalities and coronary ectasis have recently been observed in some patients (Gholamrezanezhad et al., 2007). Further, more detailed studies of larger groups of veterans and controls are now being undertaken, especially in regard to cardiovascular complications.

5.16 Recent advances in sulfur mustard poisoning and its complications

Because there are still several hundred Iranian veterans suffering from the long-term complications of SM poisoning, research of these patients is continuing. Attaran et al. (2010) have found a significant positive correlation between interleukin-6 and airflow limitation in SM-exposed patients with chronic obstructive pulmonary disease (Attaran et al., 2010). Another Iranian research group (Abolghasemi et al., 2010) has demonstrated a significant association between overall frequency of physical abnormalities in children and paternal SM exposure. They have concluded that SM exposure may have a lasting and important effect on generations to come (Abolghasemi et al., 2010).

Razavi et al. (2012) published a systematic review of the delayed complications of SM poisoning in Iranian veterans. They have reported numerous late complications among the victims and listed a wide range of respiratory, ocular, dermatological, psychological, hematological, immunological, GI, and endocrine complications, all which influence the quality of life of the exposed veterans. Although the mortality rate

attributable to SM poisoning was 3%, morbidity was high, with variety and chronicity of toxic effects and complications (Razavi et al., 2012).

Karami et al. (2013) studied the effects of delayed complications of SM poisoning on the mental health of Iranian veterans 25 years after exposure and found a wide range of psychiatric complications, including neurotic and psychotic disorders. They have recommended that psychological state should be considered more often in SM-exposed veterans and that it is important to provide more mental health centers for these patients (Karami et al., 2013).

Unfortunately, there is no antidote or effective treatment for SM poisoning. Poursaleh et al. (2012) studied the treatment of SM poisoning in Iranian veterans and have found that mustard lung has an ongoing pathological process and is an active disorder even years after exposure. They concluded that there are no curative modalities for mustard lung; therefore, primary prevention and, if injury has occurred, secondary prevention for SM victims should be considered. They recommended studies that investigate underlying physiopathology and pharmacokinetics of drugs, as well as those that conduct more surveys and controlled clinical trials to obtain more effective treatments (Poursaleh et al., 2012).

Despite two centuries of research on SM in experimental animals and in humans, the animal studies are still ongoing. Mishra et al. (2010) showed that dermal SM exposure in euthymic hairless guinea pigs induced infiltration of both $CD4^+$ and $CD8^+$ T cells into the exposed skin. They also found strong upregulated expression of pro-inflammatory cytokines and chemokines (TNF-α, IFN-γ, and IL-8) in distal tissues such as the lung and the lymph nodes. They claimed to report for the first time that SM induced a specific delayed-type hypersensitivity response that is associated with splenomegaly, lymphadenopathy, and proliferation of cells in these tissues. Their results suggest that dermal exposure to SM leads to immune activation, infiltration of T cells into the SM-exposed skin, delayed-type hypersensitivity response, and molecular imprints of inflammation in tissues distal from the site of SM exposure (Mishra et al., 2010). These immunological responses may contribute to the long-term sequelae of SM toxicity, as evident in many reports of the SM-exposed Iranian veterans (Krumbhaar and Krumbhaar, 1919; Dayhimi et al., 1988; Tabarestani et al., 1988; Zandieh et al., 1990; Ghotbi and Hassan, 2002; Mahmoudi et al., 2005).

5.17 Concluding remarks and future directions

A wide range of early and delayed toxic effects of SM can be categorized into two major groups: (1) direct toxic effects on the skin, eyes, and respiratory system with subsequent long-term complications such as chronic obstructive pulmonary disease (COPD), bronchiectasis, pulmonary fibrosis, large airway narrowing, hypopigmentation and hyperpigmentation of the skin, chronic conjunctivitis, and delayed keratitis and (2) systemic toxicities, particularly the immunohematopoietic complications, that are believed to be responsible for the increased risk of infections and malignancies in these patients. However, there are still major gaps in SM literature and further studies of human subjects who have been exposed to the agent are required. Immunological and psychological dysfunctions, as well as the relationship between SM exposure and carcinogenesis and teratogenesis, are important fields that need further investigation. Recent findings of cardiovascular complications in some of these patients also require further investigations, particularly on a molecular basis to discover the mechanisms of early and delayed toxic effects of SM in humans. Future experimental and human studies may lead to a better understanding of SM poisoning and more effective treatment.

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Chapter 6

Epidemiology of chemical warfare agents

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6.1 Introduction

While chemical warfare agents have been used in military conflict for over a century, it is only in the last three decades that increased attention has been paid to the acute and chronic health effects of these substances. Reports of poorly defined illnesses in veterans following the 1991 Gulf War—and the recent news coverage of Syrian sarin attacks—have focused international attention on the capacity of chemical agents to cause death and human suffering.

Other than investigations following the Iranian conflict in the 1980s and the Japanese terrorist attacks of the 1990s, little objective research is available on the health effects of chemical warfare agents. In particular, studies on this topic are limited by difficulties in correctly determining participants' exposures. This chapter discusses the major studies on populations exposed to chemical warfare agents and summarizes methodological issues in the literature base.

6.2 Pre-World War II

The first full-scale deployment of chemical agents occurred when Germany used them against French, Canadian, and Algerian troops in 1915 during World War I. Casualties were relatively heavy, though few deaths resulted from this initial attack. Over the course of the war, however, chemical weapons directly accounted for about 1,176,500 nonfatal casualties and 85,000 fatalities. Official records indicate that a total of 50,965 tons of pulmonary, lachrymatory, and vesicant agents were collectively deployed by both the Allies and Central Powers, including chlorine, phosgene, and mustard gas (Heller, 2005).

In 1925, 16 nations signed the Geneva Protocol, pledging never to utilize gas in warfare again; though reports of its use did follow shortly thereafter. In 1935, Italy unleashed mustard gas when invading Ethiopia during the Second Italo-Abyssinian War, resulting in 15,000 chemical casualties. While chemical agents were widely used in this and subsequent wars, there was neither formal data collection involving exposed populations, nor any systematic attempt to accurately describe the epidemiology of exposures until the 1970s.

Eventually, a longitudinal follow-up study was conducted in 1975 involving three samples of World War I veterans. Researchers sought to determine if a single exposure to mustard gas with respiratory injury increased lung cancer risk in later life (Norman, 1975). Veterans Administration death records men who served in WWI and were born between 1889 and 1893. Of these men, 2718 had been exposed to mustard gas, 1855 had been hospitalized with pneumonia in 1918, and 2578 had sustained extremity wounds rather than chemical exposures (control group). There were 4136 deaths reported across the study groups, which was 95% of that expected. Observed deaths from lung cancer numbered 69 (2.5%) for the mustard gas group, 33 (1.8%) for the pneumonia group, and 50 (1.9%) for the control group. The risk of lung cancer death among men who were gassed relative to risk for the control group was estimated at 1.3, with 95% confidence limits of 0.9−1.9.

6.3 World War II

German scientists discovered the chemical structure of sarin nerve gas in 1938 before learning the structure of soman, also a nerve agent, in the spring of 1944 (Schmaltz, 2006). Yet chemical warfare agents were not extensively used by Axis forces in battle, due in part to fear of a devastating Allied retaliation. There was, however, one account of mustard gas exposure in 1943 when Germany sunk several US ships. One of the US ships had been carrying mustard gas intended for retaliation in case of chemical hostilities by the Germans. Because the

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presence of the gas had been highly classified, authorities treating casualties ashore had no idea that they were seeing the effects of mustard gas. They prescribed improper treatments as a result. This incident was not uncovered for many years, though declassified records now show that 628 of the hospitalized casualties suffered from mustard gas exposure and 69 deaths were due, in whole or in part, to this cause (US Naval Historical Center, 2016). It was common during WWII for the impacts of gas exposures among armed and civilian populations not to be disclosed or accurately recorded. Reasons for this included military secrecy and difficulties discerning the effects of gas exposures from other types of injuries.

During the Holocaust, the Nazis used insecticide Zyklon B, containing hydrogen cyanide, to kill several million people in extermination camps. The Nazis also conducted experiments on camp prisoners using mustard gas and phosgene, and reportedly released poison gases during the Warsaw Ghetto Uprising in 1943.

A 1994 US Senate report titled, *Is Military Research Hazardous to Veterans' Health? Lessons Spanning a Half Century*, states that US military personnel were used as human subjects in 1940s chemical tests. They were exposed, without informed consent, to two agents: mustard gas and a similar compound called lewisite. The testing was reportedly done to determine how to best protect troops from the effects of chemical warfare (Pechura and Rall, 1993). This secret research, completed during WWII, involved up to 4000 men across numerous experiments. Researchers required participants to wear gas masks and clothing that had been treated to keep gases from reaching the skin. Some men were sealed in a test room for 1−4 h; others were tested in the field, restricted to an area that had been bombed with mustard gas anywhere from 1 h to 3 days prior.

In 1992, the US Department of Veterans Affairs (VA) began to allow compensation for seven conditions that can result from mustard gas exposure: laryngitis, chronic bronchitis, emphysema, asthma, chronic conjunctivitis, chronic keratitis, and corneal opacities. However, the VA expanded this list when the National Academy of Sciences published a report addressing the nonvoluntary nature of the 1940s chemical tests. It described how certain exposures were severe enough to cause burns and highlighted the lack of long-term monitoring for affected veterans. The VA updated its compensation list to include respiratory cancers (nasopharyngeal, laryngeal, and lung except for mesothelioma), skin cancer, chronic obstructive pulmonary disease, and acute nonlymphocytic leukemia (Pechura and Rall, 1993).

Bullman and Kang (2000) conducted a 50-year mortality follow-up study involving World War II Navy veterans who had experienced low-level nonlethal exposures to mustard gas. Subjects were male veterans who had participated in chamber tests at Bainbridge, MD, between 1944 and 1945; they had been wearing protective clothing and masks at the time of exposure. A control group consisted of 2663 Navy veterans who had served at the same time and location as the exposed men, but who had not participated in chamber tests. Investigators found no excess of any cause-specific mortality associated with varying doses of mustard gas that had been sufficient enough to cause skin reactions upon exposure. A strength of Bullman and Kang's study was that long-term dose—response analysis was made possible by detailed data from the original experiments; length of time in the chamber, dose of exposure, and documentation of observable acute effects were available for all subjects.

Another matter of concern was the possibility of posttraumatic stress disorder (PTSD) among chamber test participants. After randomly sampling, from the VA registry, 363 male veterans who had participated in testing, Schnurr et al. (2000) found 32% of mustard gas-exposed men to have full PTSD and 10% to have partial PTSD. Prevalence varied as a function of risk and protective factors, including volunteering, physical symptoms during the tests, and prohibited disclosure. Veterans with full PTSD reported poorer physical health, higher likelihood of several chronic illnesses and health-related disability, greater functional impairment, and higher likelihood of healthcare usage than those with no PTSD.

In a prior study, Schnurr et al. (1996) postulated that some of these diagnoses might have involved elements of "contamination stressor," in which information about the exposure—or a lack of information—acted as the stressor, rather than the incident itself. Receiving little or no information upon exposure, and then notification of potential consequences decades later could have led to vague or diffuse fear among chamber test participants. Contamination stressors often promote a future orientation—a worry about problems that might develop as a result of a past event, which can contribute to PTSD.

6.4 Post-World War II

The decades post-World War II were characterized by the development and testing of other chemicals, such as the *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX) nerve agent. By 1961, the United States was producing large amounts of VX and investing in research on the health effects of nerve agents. Military testing continued between 1951 and 1969, with various chemical and biological agents being trialed at the Dugway Proving Ground in Utah.

A large testing program took place from 1962 to 1973, when more than 5800 military personnel participated in a series of experiments on the vulnerability of warships to biological and chemical attacks. Only some of the

involved personnel consented to these tests. Many experiments involved chemical stimulants, which were thought to be harmless at the time, but are now known to have the potential for toxic effects. Test results were reported in a series of classified documents called *The SHAD Report*. In 2000, the US Department of Defense (DoD) released information about this testing along with the names of participants. This led the Institute of Medicine (IOM) to undertake a 2002 study on the potential long-term health effects of these exposures. The IOM team assembled a control group, conducted a health survey by phone, and examined mortality records. Their primary outcomes of interest were general health, medical conditions, and mortality.

The team divided SHAD participants into four groups:

- Group A consisted of 3000 participants whose exposure had been limited to either *Bacillus globigii* (BG) or methylacetoacetate (MAA).
- Group B consisted of 850 participants whose only potential exposure had been to trioctyl phosphate (TEHP); this group contained a large number of Marines.
- Group C consisted of 720 participants who had taken part in tests where active chemical warfare agents were used.
- Group D consisted of 850 subjects potentially exposed to simulants who were not in groups A, B, or C.

Control groups were assembled for comparison with each of the above groups. Of the nearly 12,500 Navy and Marine subjects, 9600 were assumed alive (i.e., no evidence of death in available records) and contacted for survey. Response rates were 60.8% for SHAD participants and 46.6% for controls. Researchers observed no differences in all-cause mortality between SHAD participants and controls, though SHAD members had a higher risk of death due to heart disease, which was statistically significant. A lack of data on participants' cardiovascular risk factors makes this difference difficult to interpret. SHAD members also reported worse overall health than controls (also statistically significant), but no specific illness patterns were identified.

Group C, the only group with potential exposure to active chemical or biological agents, reported the smallest differences in overall health compared to controls. Small differences in memory, attention, and somatization were observed among Group C versus controls. Group C participants also had higher levels of neurodegenerative conditions. There were no significant differences in self-reported hospitalizations.

The SHAD I study was significant in that it was the first epidemiological investigation of a military population with documented exposure to multiple chemical agents or stimulants. The survey was, however, conducted 30 years after the exposure and, with the exception of mortality records, was limited to self-reported measures of health. A mortality follow-up study of the SHAD cohort was published by Kang and Bullman (2009). In this study, cause-specific mortality of 4927 SHAD veterans was compared to that of 10,927 nonexposed Navy veterans of the same era. The SHAD cohort was found to have a greater risk of overall mortality, primarily from heart disease; but this heightened risk was not attributed to exposure to active chemical or biological warfare agents. This research was limited by its lack of information on participants' other potential exposures, particularly to Agent Orange (a defoliant used in Vietnam), and the possibility of error due to exposure misclassification.

In 2012, the IOM was commissioned to conduct a second epidemiological study comparing the health of SHAD veterans with that of a control population. The SHAD II study expanded on the initial investigation by reflecting a longer follow-up period and incorporating diagnostic data from Medicare and the VA. A retrospective cohort study, SHAD II involved men identified by the DoD from ship logs and personnel diaries as having been present during some portion of at least one SHAD test. The comparison population consisted of individuals who had served on ships (or a unit) of a similar class, crew complement, operating area, and home port at the time of the exposures. A small number of SHAD participants had served on five light tug boats with about 9–12 men per boat; no comparison vessels could be named for these.

The committee generated hypotheses on potential long-term health effects associated with substances used in the SHAD tests. Substance-specific exposure groups were created, including a sarin group. In addition, a broader exposure classification group was constructed to include "any chemical exposure." These groups were not mutually exclusive—a SHAD participant might have fallen into any or all of the exposure classifications. Researchers conducted a subanalysis for three distinctive groups: the crew of the *USS George Eastman*, which had participated in tests with live agents like sarin, members of the SHAD technical staff, which included persons on the light tugs, and a third group that had received unique exposure to trioctyl phosphate (TOF). TOF is an agent that was once used as a stimulant, but has come under increased scrutiny for possible carcinogenic and pulmonary effects. Each man underwent a detailed cumulative exposure assessment by the committee.

Health outcomes analyzed in relation to potential sarin exposures included systemic neurological effects (both central and peripheral nervous systems), specific neurological effects such as hearing loss, and psychological symptoms (depression, anxiety). The committee evaluated all causes of death in the study population since the 1960s, along with diagnoses assigned at hospital and

outpatient visits. Records from Medicare (1999 through 2011) and the VA Health System (late 1997 through 2011) were used to gather these data. Cause of death information was obtained from the National Death Index and death certificates found in state vital statistics offices. Ninety-one percent of the study population was identified as deceased, and 83% of SHAD participants presumed living were 65 years or older at the time of analysis.

The committee did not find any overall differences in all-cause morbidity and mortality between SHAD veterans and comparison groups. The association with heart disease mortality described in the SHAD I study was not present in this study. In fact, no notable differentials in health outcomes were identified between exposure and control groups in this extensive analysis.

The SHAD II study encountered several methodological challenges. Because of the lengthy time between the SHAD tests and this follow-up study, it was difficult to account for factors beyond the exposures that might have influenced health outcomes. Of significance, the study could not control for potential exposures to Agent Orange. The committee assumed that risk of Agent Orange exposure was similar between the SHAD and comparison groups based on evidence from VA data. Furthermore, researchers neither had access to classified data on the concentration of test substances, nor did they know ship location data for individuals, meaning exposure doses could not be estimated.

6.5 Iran–Iraq War

Iraq attacked Iran in 1980, employing mustard gas and tabun, in a war that would last 8 years. Saddam Hussein, then President of Iraq, had received chemical weapons from many countries, including the United States, West Germany, the Netherlands, the United Kingdom, France, and China (Lafayette, 2002). A total of five percent of all Iranian casualties were directly attributable to the use of such chemical agents, with Iran sustaining approximately 387 chemical attacks throughout the war (Shemirani et al., 1993). About 100,000 Iranian soldiers became chemical warfare victims, along with a significant number of civilians. In fact, nerve gas killed about 20,000 Iranian soldiers instantly during fighting. In the closing days of the war, in 1988, the Iraqi Kurdish village of Halabja was bombed with mustard gas and sarin, resulting in the death of 10% of its 50,000 residents.

Iranian scientists have produced a large volume of well-designed epidemiological studies on the health effects of chemical warfare in the decades since. Hashemian et al. (2006), for example, conducted a cross-sectional, randomized survey of 153 civilians in three towns exposed to military conflict in northwestern Iran: Oshnaviveh (low-intensity conventional warfare), Rabat (high-intensity conventional warfare), and Sardasht (both high-intensity conventional warfare and chemical weapons). The surveys measured full or partial PTSD and symptoms of anxiety and depression. Compared with those exposed to low-intensity warfare, people exposed to both high-intensity warfare and chemical weapons were at greater risk for:

- Lifetime PTSD [odds ratio (OR) 18.6; 95% confidence interval (CI), 5.8–59.4];
- Current PTSD (OR 27.4; 95% CI, 3.4–218.2);
- Increased anxiety symptoms (OR 14.6; 95% CI, 6.0–35.6);
- Increased depressive symptoms (OR 7.2; 95% CI, 3.3–15.9).

Exposure to high-intensity warfare, but not to chemical weapons, was also significantly associated with lifetime PTSD (OR 5.4; 95% CI, 1.7–17.6), compared with exposure to low-intensity conventional warfare. Further, compared with those exposed to high-intensity warfare alone, people exposed to both high-intensity warfare and chemical weapons had a greater risk of lifetime PTSD (OR, 3.4; 95% CI, 1.5–7.4), current PTSD (OR, 6.2; 95% CI, 2.0–20.1), increased anxiety symptoms (OR, 5.6; 95% CI, 2.5–12.6), and increased depressive symptoms (OR, 3.7; 95% CI, 1.8–7.2).

In a separate 2013 study, an excess of psychological symptoms were found to have persisted for Iranian victims 20 years after exposure. Roshan et al. assessed the mental health of 367 civilians who had been exposed to sulfur mustard. When compared to 128 age-matched controls, exposed people had significantly higher rates of somatization, obsessive-compulsion, depression, anxiety, hostility, and global distress indices. Another report published in 2019 described a secondary data analysis of chronic mental and physical conditions among exposed Iranian male veterans (Safi-Aghdam et al., 2019). Compared to nonexposed veterans, those who had experienced chemical attacks showed significantly elevated odds of PTSD, hypertension, coronary heart disease, and diabetes.

Sulfur mustard is rated by the International Agency for Research on Cancer (IARC) as a human carcinogen and is a known risk factor for occupational lung cancer (Nishimoto et al., 1987; Ghanei and Vosoghi, 2002). Zafarghandi et al. (2012) described the incidence of cancer in 7570 Iranian sulfur mustard-exposed veterans, versus 7595 unexposed subjects in a 25-year follow-up study. Cancer incidence was significantly higher among the sulfur mustard group, with a cancer incidence rate ratio of 1.81 (95% CI, 1.15–2.34), though no increased risk of site-specific cancer was found. The hazard ratio of cancer occurrence was 2.02 (95% CI, 1.41–2.88).

Affected Iranians have reported lasting health effects from sulfur mustard and nerve agents, even 25 years post exposure. A 2013 study by Behravan et al. substantiated these reports by revealing higher levels of DNA damage, as measured by comet assay in 25 sulfur mustard-exposed veterans, compared to controls. In 2018, these same researchers demonstrated that telomere lengths (associated in laboratory studies with aging and tumor development) in 40 sulfur mustard-exposed Iranian veterans were shorter than those observed in 40 nonexposed healthy volunteers.

The long-term respiratory effects of sulfur mustard were explored in the Sardasht-Iran Cohort Study, which included 372 exposed individuals from Sardasht and 128 nonexposed individuals from Rabat (Ghazanfari et al., 2009). As part of this research, Ghanei et al. (2010) studied pulmonary complications and found that blistering at the time of exposure was associated with more respiratory symptoms and worse lung function over time, according to computed tomography (CT) of the thorax. Blistering upon exposure was not, however, associated with air trapping, bronchiectasis, or mosaic parenchymal attenuation. Boskabady et al. (2012) built upon this research by comparing the pulmonary function of 142 exposed victims with 120 controls. A full 100% of exposed people in their sample reported respiratory symptoms. The exposed group performed worse on pulmonary function tests and also experienced respiratory symptoms with greater severity than the control group.

A possible link between sulfur mustard and cardiovascular toxicity was reported by Karbasi et al. (2013). This case-control study involved 200 consecutive patients undergoing angiographic assessment due to coronary artery disease, 100 with a history of sulfur mustard exposure and 100 without. Significantly different coronary findings were noted between the exposed and nonexposed groups, suggesting the need for more studies on the cardiovascular effects of sulfur mustard. Khosravi et al. (2018) described long-term right ventricular changes in patients exposed to sulfur mustard. As part of their study, exposed ($n = 23$) and nonexposed ($n = 19$) veterans of the Iran–Iraq War underwent echocardiographic measurements of right ventricular size and function. Pulmonary artery pressure was found to be higher and right ventricular strain lower in the exposed group. This highlights the need for more studies evaluating a possible link between sulfur mustard and cardiotoxicity.

The dermatological effects of sulfur mustard and nerve agents also have been studied among survivors of the Iran–Iraq War. Emadi et al. (2012) tracked late cutaneous complications between two exposure groups: 154 people with a history of sulfur mustard exposure and 175 people with a history of nerve agent exposure. Only 18.1% of the mustard-exposed group was asymptomatic, compared to 62.4% of the nerve agent group. Numerous mustard-induced dermatologic lesions were reported, including scars, intertrigo, xerosis, cherry angioma, hyperpigmentation, pilar keratosis, poikiloderma, and malignant tumors.

Children were exposed to toxic chemicals during this war as well. Talabani et al. (2018) were the first to evaluate the long-term health outcomes for affected children. Seventy percent of exposed children (now adults) who they surveyed reported chronic health problems, compared to 3.3% of the nonexposed population. The most common chronic problems affecting this group were visual impairments, chronic dermatological issues, and respiratory conditions, such as asthma and chronic bronchitis. The authors noted greater prevalence of these issues among people exposed during childhood than among those exposed during adulthood (determined by comparing their data with existing reports).

6.6 1991 Gulf War

Given his history of using chemical weapons against Iraqi citizens, there was much international attention that Saddam Hussein would employ these weapons against Coalition Forces. Yet the only known Gulf War exposures to anticholinesterase warfare agents did not take place during battle. Rather, one occurred during the detonation of munitions containing 8.5 metric tons of sarin/cyclosarin housed in Bunker 73 at Khamisiyah, Iraq, on March 4, 1991. The second involved the destruction of sarin/cyclosarin rockets in a pit at Khamisiyah on March 10, 1991. The US DoD reported that exposure levels were too low to activate chemical alarms or to cause symptoms at the time of detonation. However, several studies have since been conducted to assess the long-term health effects of these events. The DoD, for example, modeled the air plume that resulted from the detonation to estimate the extent of troops potentially exposed to the plume.

McCauley et al. (1999) completed a computer-assisted telephone survey of 2918 Gulf War veterans to evaluate the prevalence of self-reported medical diagnoses and hospitalizations among those potentially exposed. Respondents included veterans living in Oregon, Washington, California, North Carolina, and Georgia who had been deployed to the Southwest Asia Theater. The comparison group consisted of veterans living in the same states who had been deployed to different locations during the war. Troops reported to have served within 50 km of the Khamisiyah site did not differ from other deployed troops in terms of reported medical conditions. Hospitalization rates among exposed and nonexposed troops did not differ 9 years postwar either. Exposed troops were, however, significantly more likely than nonexposed troops to report diagnoses of high blood pressure (OR = 1.7), heart disease (OR = 2.5), slipped disk or pinched nerve (OR = 1.5), PTSD (OR = 14.9),

hospitalizations for depression (OR = 5.1), and periodontal disease (OR = 1.8). Exposed veterans also reported more diagnoses of any cancer (OR = 3.0).

Another study explored the postwar morbidity of Gulf War veterans, contrasting the health of those possibly exposed to low levels of nerve agents at Khamisiyah to that of veterans unlikely to have been exposed (Smith et al., 2003). Cox regression modeling was performed for hospitalizations from all causes, as well as hospitalizations from 15 specific diagnoses, among those on active duty from March 10, 1991 through December 31, 2000. Veterans possibly exposed to nerve agents were not found to be at greater risk for hospitalization from most chronic diseases nearly 10 years later. Only 2 of 37 models suggested that personnel possibly exposed to subclinical doses of nerve agents might be more prone to hospitalization from circulatory diseases, specifically hospitalizations due to cardiac dysrhythmias.

Bullman et al. (2005) published a mortality study of troops exposed to chemical warfare agents based on air plume models that were developed post detonation. Cause-specific mortality of 100,487 exposed US Army Gulf War veterans was compared to that of 224,480 nonexposed veterans. Most disease-related mortality rates were similar between groups. However, exposed service members had an increased risk of brain cancer death (relative risk = 1.94; 95% CI, 1.12, 3.34). This increased risk was highest among veterans who were exposed for 2 or more days versus only 1 day.

This same team conducted a separate study to examine the association between Khamisiyah plume exposure and subsequent self-reported morbidity (Page et al., 2005). Their sample included 1056 Gulf War Army veterans who had responded to the National Health Survey of Gulf War Era Veterans in 1995, and who were resurveyed in 2000. Half of the subjects had been notified of potential exposure to chemical warfare agents. Comparing notified and nonnotified subjects, there were no statistically significant differences with respect to bed days, activity limitations, clinic visits, or hospital visits. This suggested that receiving information about the potential exposure did not increase self-reports of illness and clinic visits.

Page et al. (2005) published a similar study investigating whether possible chemical warfare exposure might be associated with morbidity among Army Gulf War veterans, using data for 5555 individuals. Their analysis incorporated responses to 86 self-assessed health measures, as reported in the 1995 National Health Survey of Gulf War Era Veterans. The authors found little association between potential exposure and health outcomes after adjusting for demographic variables. They concluded that potential exposure to sarin or cyclosarin at Khamisiyah did not seem to have adversely affected self-perceived health status, as evidenced by a wide range of health measures.

The Fort Devens Cohort, part of the VA Boston Healthcare System, is one of the largest Gulf War cohorts studied to date. These veterans were initially assessed just days after their return from deployment and at additional points over the past 19 years. Reports from the early 1990s revealed excess joint pain, headaches, memory and attention difficulties, skin rashes, gastrointestinal symptoms, and sleep disorders (Krengel, 2013; Proctor, 1998; Wolfe et al., 2002). A 2019 study by Zundel et al. compared the prevalence of self-reported chronic medical conditions in the Devens Cohort ($n = 448$) to the population-based National Health and Nutrition Examination Survey (NHANES) Cohort (also self-reported). This was the fifth survey of the Devens Cohort since its inception in 2013. Participants that reported exposure to war-related chemical agents also experienced higher rates of hypertension, diabetes, arthritis, and chronic bronchitis. The authors acknowledged that while this self-reported information is useful in describing health patterns among Gulf War veterans, direct linkages could not be drawn between health status and CWA exposures.

Several neurological studies have been conducted on subgroups of the Devens Cohort. Heaton and colleagues (2007) examined the association between modeled estimates of various levels of sarin/cyclosarin exposure and volumetric measurements of gross neuroanatomical structures in 1991 Gulf War veterans. A total of 26 individuals recruited from the Devens Cohort participated (13 exposed and 13 nonexposed). Veterans underwent magnetic resonance images (MRIs) of the brain, which were analyzed using morphometric techniques. Researchers produced volumetric measurements of white matter, gray matter, right and left lateral ventricles, and cerebrospinal fluid. Volumetric data were then analyzed against exposure estimates obtained from refined models of the presumed exposure hazard area in Khamisiyah. Researchers observed no differences in volumetric measurements of discrete brain tissues between the 13 exposed and 13 nonexposed veterans. However, linear trend analyses showed a significant association between higher levels of estimated sarin/cyclosarin exposure and both reduced white matter volume (adjusted parameter estimate = 4.64, $P < .0001$) and increased volume of the right lateral ventricle (adjusted parameter estimate = 0.11, $P = .0288$) and left lateral ventricle (adjusted parameter estimate = 0.13, $P < .0001$). These findings suggest subtle, but persistent central nervous system pathology in Gulf War veterans potentially exposed to low levels of sarin/cyclosarin.

This investigative team also studied neurobehavioral performance results collected prior to the notification of veterans potentially exposed during the Khamisiyah detonation (Proctor et al., 2006). They hypothesized that exposure to sarin and cyclosarin would be associated with poorer performance on objective neurobehavioral tasks in

specific functional domains (particularly visuospatial abilities and psychomotor functioning) in a dose-dependent manner. They found that sarin and cyclosarin were significantly associated with less proficient neurobehavioral functioning on certain tasks 4–5 years after exposure. Specifically, potentially exposed veterans performed tasks requiring fine psychomotor dexterity and visuospatial abilities with less proficiency. The authors concluded that findings suggest a dose–response association between low-level sarin and cyclosarin and specific central nervous system effects.

Investigators from the University of California, San Francisco, published several additional studies on a second group of Gulf War veterans with suspected sarin/cyclosarin exposure (Chao et al., 2010). They compared the neurological health of 40 Gulf War veterans categorized as having been exposed at Khamisiyah to that of a control group with 40 nonexposed veterans. After obtaining MRI brain scans, researchers calculated volumetric measurements of gray matter, white matter, cerebrospinal fluid, and the hippocampus. They also performed a series of cognitive function tests. Exposed veterans had reduced total gray matter and hippocampal volumes compared to controls ($P < .01$). No group differences on measures of cognitive function or total white matter volume were observed. However, the authors did report significant positive correlations between total white matter volume and measures of executive function and visuospatial abilities in exposed veterans. This effect on visuospatial functioning was also displayed in a larger sample ($n = 136$) of Gulf War veterans recruited from the San Francisco area. Chao (2016) again revealed that self-reported frequency of hearing chemical alarms in theater was inversely associated with performance on a visuospatial task.

Based on these initial findings of possible low-dose sarin/cyclosarin effects, Chao and colleagues continued to examine hippocampal changes in Gulf War veterans. However, in a 2011 follow-up study, Chao et al. were not able to replicate hippocampal findings in 64 service members with possible sarin/cyclosarin exposure (Chao et al., 2011). Suspecting that imaging technique may have led to these negative findings, in a 2014 report, Chao et al. used a new technique called the automatic segmentation of hippocampal subfields to quantify hippocampal subfields in 56 exposed Gulf War veterans. (The control group included 56 nonexposed veterans). While this study revealed no difference in total hippocampal volume, differences were noted in certain subfields. The authors continued to explore potential links between chemical warfare agents (based on DoD exposure models) and hippocampal function. In 2017, they published a study on 113 Gulf War veterans who had not participated in previously published studies of hippocampal function (Chao et al., 2017). This research found exposed veterans to have smaller total hippocampal and subarea volumes compared to unexposed veterans, controlling for genetic and clinical variables. Memory performance was positively correlated with hippocampal volume.

Continuing to focus on hippocampal structural changes and neurological function, Chao et al. (2017) tested 126 previously nonstudied veterans to describe whether volume changes were accompanied with microstructural alterations of the hippocampus. Significant associations between exposure and mean diffusivity of the hippocampus were noted, and this diffusivity was related to verbal memory learning performance. These papers have been important in exploring the mechanism by which low-dose exposure to chemical warfare agents may be related to long-term and chronic alteration in hippocampal function.

6.7 Syrian War

The ongoing Syrian War, which began in 2011, has featured the use of deadly force by Bashar al-Assad's regime against dissident groups and civilians located in dissident-held territories. The use of chemical weapons against civilians. While the Syrian government denies ever having used chemical weapons, initial tests on samples from two attack sites detected chlorinated organic chemicals. Following a 2017 attack on the rebel-held area of Khan Sheikhoun, experts reported with confidence that sarin was used in the attack, which reportedly killed more than 80 people.

The most recent attack occurred in April 2018 with an estimated 1700 casualties. Citizens seen in emergency departments were described as having respiratory distress, central cyanosis, excessive oral foaming, corneal burns, and the emission of a chlorine-like odor (BBC News, 2018). Due in part to the ongoing conflict, little is known about the long-term effects these exposures might be having on targeted populations. Rodriguez-Llanes et al. (2018) applied descriptive epidemiological techniques to characterize the victims of attacks from 2011 to 2017. The authors estimated a total of 1206 direct deaths, with 1084 of those deaths resulting from five major attacks. Civilians comprised the vast majority of these deaths (97.6%), and children accounted for 13%–14% of fatalities.

In a 2015 correspondence, Hakeem and Jabri described adverse birth outcomes among women exposed to the 2013 Moadamyah chemical attacks. They reviewed the medical records of 211 pregnant women who visited the Al Ghouta Hospital in September, October, and November 2014. There were 110 women who had a self-reported exposure to the attack and 101 who had not been exposed. The miscarriage rate was 45% in the exposed group, compared to 14% in the nonexposed group.

The exposed group also had four premature births and three stillbirths, compared to none in the nonexposed group. Congenital anomalies were observed among the births, including cardiac abnormalities and multiple malformations of the head and spinal cord. The authors point to the need to closely monitor maternal records of exposed pregnant women, given their particular susceptibility and the susceptibility of their offspring.

6.8 Terrorism

Two sarin-based terrorist attacks took place in Matsumoto and Tokyo, Japan, in 1994 and 1995 respectively. These attacks killed 19 people and injured more than 6000. Morita et al. (1995) described the acute effects of the attacks, including instantaneous death by respiratory arrest in four Matsumoto victims. In Tokyo, two people died in station yards, and another 10 died in hospitals within hours to 3 months after poisoning. A total of six victims with serum cholinesterase (ChE) below 20% of the lowest normal levels were resuscitated from cardiopulmonary arrest (CPA) or coma with generalized convulsion. Of those, five recovered completely and one remained in a vegetative state due to anoxic brain damage. Electroencephalogram (EEG) abnormalities were observed for up to 5 years in some victims. Miosis and copious secretions from the respiratory and gastrointestinal tracts (muscarinic effects) were common in slightly to severely affected individuals. Weakness and muscle twitches (nicotinic effects) appeared in severely affected people. Neuropathy and ataxia impacted a small number (less than 10%) of victims; these symptoms disappeared after between 3 days and 3 months. Leukocytosis and high serum creatine kinase (CK) levels were also common. Hyperglycemia, ketonuria, low serum triglycerides, and hypokalemia were observed among those severely affected; these abnormalities were attributed to damage to the adrenal medulla.

The Matsumoto government assembled a committee of city officials, local hospital personnel, and physicians from Shinsu University to monitor immediate and long-term exposure outcomes. Their work represents the most comprehensive epidemiological studies on the acute and residual effects of chemical warfare agents to date. Three weeks after the attack, residents living within 850−1000 m of its location ($n = 2052$) were surveyed and categorized as "severely affected" if they had been admitted to the hospital, "moderately affected" if they had been treated in outpatient clinics, and "slightly affected" if they had experienced symptoms, but did not seek medical attention. At the time of the survey, 28% of affected residents remained symptomatic (69% of those severely affected, 42% of those moderately affected, and 14% of those slightly affected). The most common persistent symptoms were fatigue, dysesthesia of extremities, and ocular pain. Visual problems persisted in about 10% of severely affected victims (Yanagisawa et al., 2006).

In the Tokyo subway attack, 640 victims were seen within hours of the incident. Of these people, five were critically injured and required mechanical ventilation; another 107 were moderately injured with systemic symptoms and signs of respiratory, digestive, or neurological damage, in addition to ocular problems. A large majority (528 people) had eye damage or ocular symptoms only and were released after several hours of observation (Yanagisawa et al., 2006).

Yokoyama et al. (1998a) followed up with 18 sarin-exposed victims 6−8 months after the attack and found their mean plasma ChE to be 72.1 (normal range = 100−250 IU/L). In neurobehavioral testing, this group scored significantly lower on the Digit Symbol Substitution Test, a timed, paper-and-pencil cognitive test, than the control group. Exposed people were also more likely to have higher scores on both the General Health Questionnaire, an indication of psychological distress, and fatigue measures than controls; their PTSD scores were also greater. Furthermore, victims displayed poorer postural balance, suggesting their integration of visual input might have been impaired (Yokoyama et al., 1998b). P300 and VEP (P100) latencies were significantly prolonged for sarin victims compared with matched controls (Murata et al., 1997). Additionally, CVRR (electrocardiographic R−R interval variability) among victims was significantly related to serum ChE levels measured immediately after sarin exposure. While people exposed to sarin had high PTSD scores, those scores were not significantly associated with any specific neurophysiological data. These findings suggest that asymptomatic aftereffects from sarin, rather than PTSD, persist in the higher and visual nervous systems beyond the turnover period of ChE.

The National Police Academy in Japan (1999) conducted a survey of 1247 Tokyo residents who had reported a sarin exposure to police at the time of the incident. In the survey, more than half complained of physical symptoms, such as asthenopia and decreased visual acuity; 17% reported lasting psychological trauma from the event. Fourteen percent remained unable to ride on subways 3 years after the incident.

There have been several follow-up studies describing residual effects from the Tokyo subway exposures. Ohtani et al. (2004) followed 34 victims for 5 years after the event. Not only PTSD, but also nonspecific mental symptoms, continued to affect them at high rates. A total of 11 victims were diagnosed with current or lifetime PTSD. Those with PTSD showed higher anxiety levels and greater degrees of visual memory impairment.

Yamasue et al. (2007) conducted a study 5 years post exposure to identify persistent morphological changes in

Tokyo subway attack survivors. In this study, 38 people who had been treated in the emergency department for sarin intoxication and 76 control subjects underwent weighted and diffusion tensor MRIs. Researchers compared victims' present-day ChE levels to their levels immediately after the attack. Voxel-based morphometry exhibited smaller-than-normal regional brain volumes in the insular cortex and neighboring white matter, as well as in the hippocampus. Reduced regional white matter volume correlated with both decreased serum cholinesterase levels and the severity of chronic somatic complaints related to interoceptive awareness. Furthermore, voxel-based analysis of diffusion tensor MRIs demonstrated a significantly lower-than-normal fractional anisotropy in the victims. These findings suggest that sarin intoxication might be associated with structural changes in specific brain regions.

Attention also has been paid to rescue and safety workers who responded to the Tokyo attack. Nishiwaki et al. (2001) looked at 27 male rescue team members and 30 police officers 3–45 months after the event. The subjects showed decreased performance on the Digit Span Test, a measure of short-term memory retention. However, they noted no effects on participants' stabilometry or vibration perception thresholds. In another study, Li et al. (2004) followed 27 male firefighters and 25 male police officers 3 years after the attack for genotoxic effects. They found an elevated frequency of sister chromatid exchanges in the victims' lymphocytes, which were related to the percentage of ChE inhibition observed just after the attack.

6.9 Concluding remarks and future directions

This chapter described the major epidemiological studies of populations exposed to chemical warfare agents. Many of the studies involving military populations took place decades after the exposure and suffer from inaccurate exposure assessment, as well as a lack of clinical data. However, in the past two decades, studies surrounding terrorist attacks and the Iran–Iraq War have provided comprehensive data on the scope of health outcomes associated with warfare chemicals. Emerging reports of chronic disease in the years post exposure point to the need for long-term follow-up studies involving victims of such events. As more women participate in military forces and public safety units, studies are needed to document gender-related health effects. Chemical warfare exposure continues to be a threat in the current Civil War in Syria. More studies are needed to determine the effects of these agents on vulnerable populations, such as children and pregnant women.

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Chapter 7

Chemical weapons of mass destruction and terrorism: a threat analysis

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7.1 Introduction

After the September 11, 2001 (9/11) terrorist attacks in the United States, the high risk of possible attacks with chemical weapons (CWs), especially by groups linked to jihadist terrorism, has been perceived. Before 9/11, in 1994 and 1995, Aum Shinrikyo, a religious organization in Japan, used sarin (a nerve agent) in attacks in Matsumoto and on the Tokyo subway, causing a large number of casualties. These terrorist attacks had a large impact on the international chemical defense and intelligence communities, but not on other circles, perhaps because a chemical attack by a religious organization in Japan seemed something far removed from the reality of the rest of the world. But this changed after 9/11, when the mailing of letters containing *Bacillus anthracis* spores accompanied by images of the attacks on New York City's World Trade Center towers increased the concern about weapons of mass destruction (WMD) attacks, including attacks with CWs.

In this chapter, information is analyzed from open sources regarding the possible use of CWs by terrorist groups, especially by those affiliated with the jihadist terrorism network. As religious terrorist groups, Al Qaeda and Daesh do not fit the assumption made by Brian Jenkins in 1975 that "terrorists want a lot of people watching and a lot of people listening, and not a lot of people dead" (Jenkins, 1975). This statement fits better with secular terrorist groups. But for religious terrorist groups, "divine duty" results in disappearance of moral restraints that would justify "a lot of people dead" in their terrorist attacks, such as the 9/11 attacks. And if CWs are part of the WMD concept because they can cause a large number of casualties, they could be very good tools for jihadist terrorist groups to achieve their goals.

7.2 Chemical weapons for terrorist actions

7.2.1 "Classical" chemical warfare agents: vesicants and nerve agents

The two main chemical warfare agents (CWAs) are vesicants and nerve agents (Table 7.1). The first chemicals used as weapons in World War I included toxic industrial chemicals (TICs) that were widely available in the chemical industry. These included lung-damaging agents like chlorine and phosgene, as well as cyanides that were named "blood agents." When these agents proved to be effective at the tactical level, research and development programs of CWAs started (Pita, 2008). These programs tried to obtain chemicals that would be effective in battle because of their physicochemical and toxicological properties, although they had no use in the industrial field.

First, sulfur mustard, a vesicant warfare agent, was produced and used in World War I. Mustards alkylate a wide range of biologically important molecules producing cytostatic, mutagenic, and cytotoxic effects. In 1936, just before the start of World War II, tabun was synthesized and then produced as the first nerve CWA. Nerve agents inhibit acetylcholinesterase (AChE) throughout the body, causing an accumulation of acetylcholine (ACh), which produces overstimulation at muscarinic and nicotinic receptors in the peripheral and central nervous systems.

Vesicant agents (sulfur mustards, nitrogen mustards, and lewisites) and nerve agents (tabun, sarin, soman, and VX, among others) are included in the Chemical Weapons Convention (CWC) Schedule 1 of chemicals for the application of verification measures. This schedule features chemicals that have little or no use in the industry and have been developed, produced, stockpiled, or used

Handbook of Toxicology of Chemical Warfare Agents. DOI: https://doi.org/10.1016/B978-0-12-819090-6.00007-6

TABLE 7.1 Some potential chemical agents for terrorist actions.

Vesicants	Sulfur mustards
	Nitrogen mustards
	Lewisites
Nerve agents	Tabun (GA)
	Sarin (GB)
	Soman (GD)
	VX
	"Novichok" agents
Incapacitating agents	BZ
	Opioids
Riot control agents	CS
	CN
	CR
	Capsaicin
Toxic industrial chemicals	Chlorine
	Phosgene
	Cyanides
	Phosphine
	Hydrogen sulfide
Toxins	Ricin

as CWs. Other nerve agents include intermediate volatility agents (IVAs) like GV, as well as those allegedly developed in the Foliant program of the former Soviet Union during the Cold War, commonly known as Novichok ("Newcomer") (Nepovimova and Kuca, 2018a; Pita, 2008). One of the most interesting properties of the Novichok agents is that they were not included until 2020 in the CWC's schedules for the application of verification measures. Most of the public information available about Novichok agents comes from Vil Mirzayanov, an analytical chemist who worked in the Foliant program. In his book *State Secrets*, Mirzayanov (2008) published for the first time the chemical structures of some of the agents developed.

Nerve agents can be considered as the most important CWAs because of their high toxicity and their high versatility for tactical use. This high versatility is attributable to the fact that although all nerve agents are liquid at room temperature, some can be considered persistent agents (e.g., VX) because of their low volatility, whereas others can be considered nonpersistent (e.g., sarin) because their volatility is higher. Persistent agents would be useful against targets with no occupational interest that would be contaminated for a certain period of time. In contrast, nonpersistent agents would evaporate faster and would be the choice against targets that need to be occupied.

Toxicological and physicochemical properties of nerve agents would also make this group the CWs of choice for terrorist groups. However, the synthesis process requires some level of expertise and is far more complex than the recipes featured in the "do it yourself" (DIY) manuals available on the Internet (Fig. 7.1). Even Aum Shinrikyo—which had excellent financial resources and personnel with the required expertise and performed their attacks before the entry into force of the CWC—had some difficulties in synthesizing sarin (Tu, 2002).

7.2.2 Incapacitating agents

Incapacitating agents are chemicals that produce a disabling condition that persists for hours to days after exposure has occurred. These were studied during the Cold War when it was assumed that incapacitating the enemy would cause damage not only because casualties would become unavailable for duty but also because they would need to be evacuated and would consume more logistical resources (Pita, 2008). Incapacitating agents include depressants and stimulants of the central nervous system (CNS). The main incapacitating agent is BZ (3-quinuclidinyl benzilate), a CNS depressant included in Schedule 2 of the CWC because it is produced in small commercial quantities for biomedical purposes.

Opioids have also been studied as incapacitating CWAs. In October 2002, a fentanyl derivative was used by Russian Special Forces to end a hostage crisis during which Chechen terrorists had taken approximately 800 hostages at a Moscow theater (Wax et al., 2003). The dissemination of this opioid through the theater ventilation system killed approximately 130 people and injured more than 600. The fact that medical services did not have information about the identity of the chemical resulted in a lack of antidotal treatment of the poisoned hostages.

7.2.3 Riot control agents

Riot control agents (RCAs), popularly referred to as "tear gas" or "pepper spray," should not be confused with incapacitating agents. Unlike the latter, RCAs are local irritants with disabling effects that disappear within a short time after the exposure. Most important, RCAs do not act at the CNS level; therefore, they have lower toxicity and a wide margin of safety when compared with incapacitating agents. RCAs are not listed in the CWC schedules. Although the Convention prohibits its use as a method of warfare, it does not prohibit its use for law enforcement purposes.

FIGURE 7.1 A jihadist manual dealing with the production of CWs available on the Internet.

7.2.4 Toxic industrial chemicals

TICs are of special interest in the chemical terrorism context because they are widely available in large quantities. An attack against a chemical plant or transport vehicle may result in a release with potentially catastrophic consequences, like the release of methyl isocyanate in Bhopal (India) in 1984.

The importance of TICs is reflected in the fact that the CWC features a schedule that includes chemicals that may be produced in large commercial quantities for industrial purposes, but that have been produced, stockpiled, or used as CWs. Schedule 3 includes phosgene, a lung-damaging agent, and some cyanides. Cyanides are commonly named "blood agents," something that may cause confusion, leading one to think that cyanides affect the oxygen transportation in the blood. The reason for calling cyanides blood agents was because chlorine and phosgene were toxic chemicals with local effects in the respiratory tract, whereas cyanides had to be absorbed in the lungs and passed to the blood to produce systemic effects. Although cyanides produce cellular asphyxiation at the mitochondrial level, it is not rare to see them as "blood agents" when mentioned in the CWs or chemical terrorism context.

7.2.5 Toxins

Toxins are chemical substances of biological origin, although synthesis procedures for some nonprotein toxins that have been studied as weapons are widely available (Jacobi et al., 1984; Tanino et al., 1977). Toxins are included in the CWC because it covers toxic chemicals "regardless of their origin or of their method of production." Actually, two toxins, ricin and saxitoxin, are explicitly included in Schedule 1 of the CWC. However, toxins are also included in the Biological and Toxin Weapons. For these reasons, toxins can be considered CWs, biological weapons, or mid-spectrum agents.

7.3 Tampering with chemical weapons

Throughout history, there have been extortion tampering cases where toxic chemicals have been used. For example, at the end of 1982, there were seven fatal cases of cyanide poisoning after the ingestion of acetaminophen-tampered capsules (Dunea, 1983; Wolnik et al., 1984). McNeil Consumer Products had to destroy approximately 22 million units and changed the production from capsules to tablets that were more difficult to tamper with. Those responsible were never identified, and some imitators in the US started contaminating medicines and food products with toxic chemical substances.

In some cases, blackmailers have even attacked governments. For instance, on March 2, 1989, an anonymous person called the United States and Japanese embassies in Santiago, Chile, alerting them that cyanide had been injected in fruit destined to be exported to those countries (Grigg and Modeland, 1989; Spiers, 2000; Wilkening, 1999). Although countermeasures were not taken initially, after a second call to the US embassy on March 17, the US Food and Drug Administration (FDA) decided to start inspections at the Philadelphia port, the port of entry of

approximately 80% of the fruit from Chile. After an extensive search, only two grapes that had been injected with a small quantity of sodium cyanide were found. However, as a preventive measure, on March 13, all fruit coming from Chile was placed on quarantine. Subsequent searches did not find more tampered fruit and the quarantine ended on March 17. Although the event ended without any poisoning cases, it meant losses of approximately $300 million for Chile's fruit export market.

Europol's 2018 terrorism situation and trend report mentions that between 2013 and 2017 anarchist organizations in Greece and Italy threatened to intentionally contaminate food or water (Europol, 2018). These threats were part of campaigns to target multinational corporations in order to produce loss of revenue.

7.4 State terrorism

CWs have also been used in the assassination of selective targets by secret services. The defection of the KGB's Capt. Nikolai Khohhlov in February 1954 brought to light some of the devices created for this type of attack. In fact, Khohhlov defected in the middle of a mission to assassinate Georgi Okolovich, a Soviet dissident living in West Germany, by using a cigarette pack that concealed a hydrogen cyanide ampoule (Andrew and Gordievsky, 1990). Similar cases include the killing of two Ukrainian exiles in Munich in 1957 and 1959 by a KGB agent who used a special gun device to disperse hydrogen cyanide. According to Vadim Birstein, since the 1920s and at least until the late 1970s, different laboratories in the Soviet Union were in charge of the development of assassination devices for their Secret Services (Birnstein, 2001).

One of the most bizarre stories of assassination is the murder of Georgi Markov, a Bulgarian journalist exiled in the United Kingdom, with an umbrella that delivered ricin (Crompton and Gall, 1980; Knight, 1979). The umbrella was allegedly modified to fire a small pellet filled with approximately 500 μg of ricin. On September 7, 1978, Markov was waiting for a bus on Waterloo Bridge when he felt a strong puncture in the back of his right thigh. When he turned around, a man with an umbrella apologized. The next day he was hospitalized with fever, vomiting, speaking difficulties, and a white cell count of 10,600/mm^3 that increased to 33,200/mm^3 3 days later. On September 11, Markov died of cardiac failure. The autopsy revealed the presence of a small spherical pellet with a diameter of 1.53 mm that had two holes of 0.34 mm. Days before Markov was attacked, on August 26, another Bulgarian exiled in Paris (France), Vladimir Kostov, also felt a puncture in his back and had to be hospitalized for 12 days, although he finally recovered. On September 26, a similar pellet—spherical, of 1.52 mm diameter, with two holes of 0.34 mm—was extracted from his back.

No public reports about the identification of ricin have been published. The ricin link was made based on the differential diagnosis by the UK's Ministry of Defense scientists (they even administered ricin to a pig to compare the clinical signs of the poisoning with those observed in Markov), histopathological findings that were similar to those found in different *in vivo* tests with animal models, and intelligence regarding a ricin program in the Soviet Union and perhaps other Warsaw Pact nations (Birnstein, 2001; Waller et al., 1966).

Years later, former KGB members stated that the umbrella was provided by the KGB to the Bulgarian Secret Services, and that previous attempts to kill Markov had been unsuccessful (Andrew and Gordievsky, 1990; Kalugin, 1994). Because Bulgarian documents about the Markov case appear to have been destroyed, only future KGB document releases would disclose more information about this murder (Carus, 2002).

More recently, on March 4, 2018, former Russian double agent Sergei Skripal and his daughter were found unconscious in Salisbury (UK) and transported to hospital where they were treated for nerve agent poisoning (Vale et al., 2018). A police officer who responded to the incident and visited Skripal's house was also poisoned and received similar treatment. The three patients survived and were eventually discharged from hospital. Analyses of environmental and biomedical samples by Porton Down's Defence Science and Technology Laboratory (DSTL) and by the Organization for the Prohibition of Chemical Weapon (OPCW)'s designated laboratories, identified a Novichok nerve agent of high purity (Bristow, 2018; Johnson, 2018; Sedwill, 2018; Wilson, 2018). Russia's ambassador to the UK leaked the agent identity as "A-234" (Faulconbridge and Holden, 2018). The highest concentrations were found in samples taken from the handle of Skripal's front door, indicating that most probably exposure was caused by dermal contact with a liquid agent applied to the handle. After the analyses results were made public, the British government stated that based on capabilities and motivations, it was "highly likely" that Russia was responsible for the attack (Bristow, 2018; Johnson, 2018; Sedwill, 2018). The Russian authorities denied any involvement in the Salisbury incident and the existence of a chemical program that developed Novichok agents (Embassy of the Russian Federation to the United Kingdom, 2019).

On June 30, a man and a woman from Amesbury (about 7 miles north of Salisbury) were hospitalized and treated for nerve agent poisoning after handling a perfume bottle they had found at Queen Elizabeth Gardens in Salisbury. The man was subsequently discharged from

hospital, but the woman died on July 7. The same Novichok agent was identified in biomedical and environmental samples analyzed by Porton Down's DSTL and OPCW's designated laboratories (OPCW, 2018). The analysis of the fake Nina Ricci perfume bottle content showed a concentration of 97%–98%. Therefore, the perfume bottle had most probably been used in the attack on Skripal and then discarded.

In early September, British authorities announced that they had identified two suspects who were linked to the Russian military intelligence service (GRU) (Bellingcat Investigation Team, 2018a,b,c,d,e). The Salisbury incident, as well as the 2004 dioxin poisoning of Viktor Yushchenko, former Ukrainian president, and the 2006 polonium-210 poisoning of Alexander Litvinenko show that assassination of selected targets with CWAs is not something of the past (le Polain de Waroux et al., 2011; Sorg et al., 2009).

The former Warsaw Pact Secret Services were not the only ones to perform such CW programs, as seen during the appearances of CIA officials in the Senate Committee known as the Church Committee, which took place during September 16–18, 1975. Former CIA Director William Colby explained that the CIA had an incapacitating agents' program (known as MKULTRA) and provided documents detailing the CIA stock of CWs at that time, which included small quantities of BZ, toxins, and cyanide pills, among others (USS, 1976).

On February 13, 2017, Kim Jong-nam, half-brother of North Korea's leader, Kim Jong-un, was killed at Kuala Lumpur International Airport (Malaysia) by two women who applied VX to his face (Nakagawa and Tu, 2018; Nepovimova and Kuca, 2018b). It is still not clear if VX or a binary system was used for the attack. The North Korean intelligence service was blamed for the assassination, although North Korea has denied responsibility (Swenson, 2017).

7.5 Nationalist and separatist terrorist groups

Secular terrorist groups have not shown significant interest in CWs, perhaps thinking that its use may be rejected by their own followers (although many of them have performed terrorist attacks with conventional explosives that produced a high number of casualties). There are some reported cases indicating that nationalist and independent groups have used chemical substances in sabotage actions. For example, in 1992 the Kurdistan Workers' Party (PKK) allegedly contaminated several water tanks with sodium cyanide in an air base near Istanbul (Karasik, 2002). The attack was aborted when the recipients of 25 kg of potassium cyanide were found next to the tanks.

Another example can be found in the Liberation Tigers of Tamil Eelam (LTTE) in Sri Lanka. Although the LTTE was a secular terrorist group, their members showed a level of veneration for their leader, Velupillai Prabhakaran, akin to that of a religious cult, and even used tactics used by jihadist terrorist groups. LTTE members were known to carry sodium cyanide capsules that were to be used to commit suicide in case of capture. There are also some reports about the use of sodium cyanide against Sri Lanka's economic interests (Carus, 2002; Wilkening, 1999). The first report was from 1986, which mentions attempts to sabotage tea exporting by informing different embassies that some lots had been contaminated with cyanide. US authorities analyzed different lots but did not find anything suspicious. The second report was from December 1996, when potassium cyanide was allegedly applied to stamps used by the Sri Lanka Army. However, the most important LTTE chemical attack occurred in June 1990, when drums of chlorine were used against a military camp located in east Kiran, although there is no information about the number of casualties (Hoffman, 2009).

7.6 Left-wing terrorist groups

In May 1975, the Baader-Meinhof Gang, also known as the Red Army Faction (RAF), a left-wing terrorist group based in West Germany, threatened to use sulfur mustard in Stuttgart and other cities in Germany if imprisoned members were not freed. However, in-depth studies of this incident have found no evidence of the RAF having access to sulfur mustard (Claridge, 2000). The terrorist organization was taking advantage of media reports indicating that old sulfur mustard containers from World War I had disappeared from a CW destruction facility.

Colombian guerrillas (initially left-wing terrorist groups but today considered to be narcoterrorism groups because of their association with organizations that deal with the illicit traffic of drugs of abuse) have performed small attacks with CWs. On December 2, 2001, members of the Revolutionary Armed Forces of Colombia (FARC) attacked a police station in San Adolfo (Huila), probably with cyanogen chloride, killing four policemen (Pita, 2008). Since then, munitions charged with cyanide have been found in raids against FARC camps. In fact, in November 2007, Colombia's National Police showed their concern after finding a clandestine FARC laboratory near the frontier with Ecuador that was working on the filling of rockets with toxic chemicals. The chemicals included ammonia, chlorine, and cyanide compounds.

7.7 Right-wing terrorist groups and lone actors

Koehler and Popella (2017) consider the possibility that far-right domestic terrorism may be tempted to use WMD in what could be considered as a competition with jihadist terrorism to gain public and media attention. In fact, it is not uncommon to find criminal activities using CWs linked with right-wing groups or sympathizer lone actors. The two most common agents used have been cyanides and ricin. In fact, hydrogen cyanide production and ricin extraction procedures are commonly found in "cookbooks," publications that are popular among members of white supremacist groups and in "amateur terrorist" circles. Some of these titles include *Assorted Nasties*, *The Poisoner's Handbook*, *The Preparatory Manual of Chemical Warfare Agents*, and *Silent Death*.

Although hydrogen cyanide gas (boiling point at 760 mmHg, 25.7°C; vapor pressure at 20°C, 600 mmHg) is easy to obtain by mixing the right cyanide salt and acid, transporting and mixing the reagents without being discovered constitutes one of the biggest hurdles to terrorist attacks. Regarding the ricin extracted with procedures included in cookbooks, it is not able to achieve a product capable of causing a large number of casualties by any exposure route, mainly because of the low content of toxin in the final extracts (Pita et al., 2004).

In the mid-1980s, a group named the Covenant, the Sword, and the Arm of the Lord obtained potassium cyanide to contaminate water supplies in different US cities (Stern, 2000). In a raid that took place on one of their facilities, authorities found a drum with 30 gal of potassium cyanide. When one of its members was told that the attack would kill not only those he considered enemies (Jews and "mud-people") but also other people, including members or sympathizers of the group, his reply was: "We felt that God would take care of this [and] that those who were meant to die would be poisoned" (Stern, 2000). And when he was told that 30 gal were not enough to obtain a toxic concentration in the city's water reservoir he answered: "God would... make sure the poison got to the town" (Stern, 2000). He even explained that they had decided to act because there were signs of the arrival of the Armageddon: "You get tired of waiting for what you think God is planning" (Stern, 2000). These statements make it clear that terrorist groups with religious motivations often have no moral restraints in performing attacks that could cause a large number of casualties. In fact, Christian-identity right-wing groups and lone actors have also carried out terrorist attacks that have caused a large number of casualties, such as the April 19, 1995, bombing of the Alfred P. Murrah Federal building in Oklahoma that caused 168 fatalities and more than 500 nonlethal casualties.

In 1991, four members of the Minnesota Patriots Council, an antigovernment right-wing group, obtained a kit called "Silent Tool of Justice" that contained approximately one dozen castor plant seeds and a toxin extraction procedure that one of the members followed (Tucker and Pate, 2000). The plan was to mix the toxin with dimethyl sulfoxide (DMSO) and *Aloe vera* gel and apply it on doorknobs of different individuals that were considered enemies of the group. The four members were arrested in 1994.

Cases of lone actors with intentions of using CWs are not rare. For example, in the mid-1970s, authorities found 25 pounds of sodium cyanide at the apartment of Muharem Kurbegovic, the "Alphabet Bomber" (Simon, 2019). Other chemicals and documents found showed a special interest in CWs. Kurbegovic had threatened to use nerve agents, but he did not have any when arrested. A good example of a lone actor linked to right-wing groups is Thomas Leahy (Carus, 2002). In 1997, an enforcement officer in Wisconsin responded to a report of spousal abuse made by Leahy's wife. When the officer arrived, the wife opened the door wearing a mask and explained the reason to the officer: "Because my husband is in the basement making poison gas" (Gurr and Cole, 2000). Among the items found in the laboratory were some cookbooks, ricin extracts, and three spray bottles with a mix of nicotine and DMSO. Also, in 2004, an arms trader with white supremacist group connections was sentenced to 11 years in prison (Kosal, 2006). In April 2003, authorities had raided his Texas warehouse arsenal and found that he was building an improvised chemical device (ICD) based on the mixing of sodium cyanide and an acid.

7.8 Apocalyptic cults: Aum Shinrikyo

Aum Shinrikyo's sarin and VX attacks are detailed in Chapter 4, Sarin attacks in Japan: acute and delayed health effects in survivors. This Japanese cult is a clear example of how difficult it is to produce a CWA and use it as a weapon with an efficient dissemination system, especially considering that they had adequate financial resources and technological means. The cult also benefited from the 1951 Religious Corporation Law that grants tax exemptions to religious organizations in Japan and protection against possible interference of the state in their activities. This advantageous situation allowed Aum Shinrikyo, when it started its chemical, biological, and toxin programs, to enjoy a position that would be similar to that within a proliferating state—where there is no need to hide these activities from the security forces because the program is integrated within government activities—rather than a terrorist organization.

But sarin and VX were not the only CWs used by the cult. After the Tokyo sarin terrorist attacks in 1995, Aum Shinrikyo cult members tried an attack with hydrogen cyanide. On May 5, they used an ICD in Shinjuku's Tokyo subway station that consisted of two plastic bags, one with sodium cyanide suspended in 2 L of water and another with 1.5 L of a sulfuric acid solution (Dolnik and Gunaratna, 2008; Tu, 2002). The activation system consisted of two condoms, one with sodium chlorate and another with sulfuric acid, so when the latter destroyed the latex the fire produced would break the plastic bags and allow the mixing of the cyanide salt and the acid, producing hydrogen cyanide. However, the quantities were not well-calculated, and the fire destroyed the ICD. On July 4 and 5, another two attacks occurred in Tokyo, this time using an ICD in which the activation of an electrical device with blades would break the plastic bags. One ICD failed, while the other only produced one case of mild poisoning.

7.9 Jihadist terrorism: Al Qaeda, Daesh, and the Global Jihad Movement

7.9.1 Weapons of mass destruction intentions

There are three phases in the statements of Al Qaeda members related to WMD. In the first phase, Al Qaeda tended to justify the acquisition and possession of these weapons from the point of view of deterrence. This phase goes as far back as 1998, when Osama bin Laden had stated that acquiring WMD was a "religious duty" (Pita, 2007). This and similar statements were made by bin Laden in different interviews after the US attack on the Al Shifa Pharmaceutical Industries factory in Khartoum on August 20, 1998. This attack was part of Operation Infinite Reach in retaliation for the bombings of the US embassies in Kenya and Tanzania on August 7, 1998, for which the bin Laden terrorist network was blamed by US officials. The Al Shifa target was justified in the finding of *O*-ethyl methylphosphonothionate, a precursor of the nerve agent VX, in soil samples outside the factory, and in the financial contributions of bin Laden to the production of CWs. Soon after 9/11 and the mailings of envelopes with *B. anthracis* spores in the United States, bin Laden was interviewed again. When asked about reports claiming that he was trying to acquire WMD, he answered: "We have the weapons as deterrent" (Pita, 2007).

The second phase of statements of Al Qaeda members related to WMD began soon after the overthrow of the Taliban regime in Afghanistan. Al Qaeda's reasoning was that the Coalition Forces had used conventional weapons (e.g., missiles) that had caused a large number of casualties and destruction, and for this reason these weapons could be considered WMD. This interpretation justified the use of chemical, biological, radiological, and nuclear (CBRN) weapons as retaliation for similar attacks. The most well-known statement in this second phase was made by Suleiman Abu Gheith, Al Qaeda's spokesman, who wrote in his 2002 electronic article "In the Shadow of the Lances" that based on this reasoning they had the "right to kill four million Americans—two million of them children—and to exile twice as many and wound and cripple hundreds of thousands" (Pita, 2007).

The third phase started in May 2003, when Nasir bin Hamad Al Fahd, a Saudi cleric who supports the global jihad movement, issued a fatwa justifying and authorizing the use of WMD (Paz, 2005). Al Fahd used arguments based on reciprocity, stating that the US had used weapons that caused a large number of casualties and mass destruction. But what was new in this fatwa was that Al Fahd's arguments were also based on Islamic texts that supposedly justify that it is permissible to use WMD if those engaged in jihad decide there is benefit in using them. And this is the case of Al Qaeda's influential strategist Mustafa Setmarian Nasar, better known as Abu Musab Al Suri, who posted a letter on the Internet in December 2004 stating that the use of WMD was "a necessity." Al Fahd's arguments were recovered in 2008, when Ayman Al Zawahiri published "Exoneration." Moreover, in 2015 Al Fahd allegedly pledged allegiance to Abu Bakr Al Baghdadi, leader of Daesh (Dean et al., 2018; Johnson, 2015).

Al Qaeda's speech about WMD has been adopted in Daesh's doctrine, which is not strange because of the link between these terrorist organizations. In fact, Al Qaeda in Iraq (AQI), founded by Abu Musab Al Zarqawi, can be considered the embryo of Daesh. Al Zarqawi was heavily influenced by Abu Abdullah Al Muhajir, an Egyptian who was the head of Al Qaeda's Religious and Scholarly Committee (Abu Hanieh and Abu Rumman, 2015; Dean et al., 2018; Winter and Al-Saud, 2016). Al Muhajir wrote *The Jurisprudence of Blood*, copies of which started circulating in AQI training camps in Iraq in 2004 (Dean et al., 2018). In this book, the use of WMD is justified as a more effective tactic than suicide bombings to cause mass casualties (Al Ansari, 2018). The book also justifies killings of noncombatants, beheadings, and hostage operations, among others. Al Zarqawi used its texts to justify AQI indiscriminate killings in Iraq. *The Jurisprudence of Blood* is still considered a key publication in Daesh and it is used for ideological indoctrination purposes (Al Ansari, 2018; Townsend, 2018).

Jihadist terrorism's interest in WMD may also be based on their important psychological effects. An objective of using these weapons in a military scenario is not only to cause physical casualties but also to demoralize troops. Similarly, in a terrorist attack on civilians, one of

the primary goals is to create a general sense of panic and fear, resulting in psychological trauma and disruption to economic and social activities (Zanders, 2003). For these reasons, WMD can also be regarded as "weapons of mass *disruption*." For instance, it is frequently asserted that the Aum Shinrikyo sarin subway attack caused more than 5000 casualties, but only approximately 1000 patients had clinical signs of sarin exposure (Woodall, 1997). That means that approximately 4000 people who sought attention in medical facilities were mainly psychological casualties with psychogenic symptoms. Based on a book by Abu Walid Al Misri, editor of a magazine for the Taliban, quoted in Peter Bergen's book *The Osama bin Laden I Know* (2006), Al Qaeda has been aware of the psychological effects of WMD since they first thought about acquiring them.

7.9.2 Chemical weapon capabilities

7.9.2.1 Al Qaeda

The first information of Al Qaeda's CW programs came from Jamal Ahmed Al Fadl (an Al Qaeda member who defected and became a United States government informer in 1996), who claimed that in the early 1990s he and other Al Qaeda members discussed the start of a manufacturing program of CWs with a Sudanese army officer (Bergen, 2001; Clarke, 2004; Tucker, 2006). In fact, the Al Shifa facility would have been part of this program.

Since October 2001, reporters and military forces in Afghanistan found written and electronic documents with rudimentary procedures for the production and use of toxic chemicals (Pita, 2007). These procedures are similar and, in some cases, word-for-word translations from those included in the cookbook publications mentioned previously. Two well-known cookbooks were found in Afghanistan, *Assorted Nasties* and *The Poisoner's Handbook*. This material came mostly from the Abu Khabab camp located in the Darunta training camp complex that specialized in explosives, but also in toxic chemicals (Dean et al., 2018; Gunaratna and Acharya, 2006). This camp was named after the man who ran it, the Egyptian Midhat Mursi, commonly known as Abu Khabab Al Masri, who was killed in a US air strike on the Pakistan−Afghan border in July 2008.

Ahmed Ressam, an Algerian arrested by US authorities for carrying explosives that he intended to use in a bombing against the Los Angeles International Airport, explained in court in July 2001 that he had been trained in the Darunta training camp complex in 1998 to prepare hydrogen cyanide by mixing a cyanide salt and sulfuric acid (Pita, 2007). He was told to release it near the air intake vents of buildings and even participated in live training exercises using dogs. In August 2002, CNN aired several Al Qaeda videotapes obtained in Afghanistan that revealed experiments with chemical agents on dogs. One of those tapes showed several men—apparently after having mixed several chemical reagents—rushing out of an enclosure, inside which was a tied-up dog. Soon, a white vapor appeared and a few moments later the dog started showing the first clinical signs of exposure. The quality of the images was not good enough to identify a toxidrome, but the videotape was reminiscent of what Ahmed Ressam said about his training with hydrogen cyanide in Afghan camps. These experiments were allegedly recorded by Abu Khabab in the Darunta training camp complex.

In November 2006, a book published by Nasiri (2006), the pseudonym of an alleged informer of the British and French intelligence services, explained his participation in experiments with animals using cyanide at the Khalden training camp. Nasiri also describes the failed attempts to develop sulfur mustard munitions in Khalden. He states that after many trials, finally the camp members celebrated when the shell produced a "thick cloud of smoke." However, this does not mean that the munition was effective in disseminating sulfur mustard.

More recently, Aimen Dean, a former MI6 spy inside Al Qaeda who was trained and worked with Abu Khabab between 1997 and 1999, confirmed the animal experiments in Darunta, especially with cyanides, but also with chlorine, nicotine, phosgene, and phosphine (Dean et al., 2018). Khabab obtained chemicals for his camp through the university of Jalalabad. Dean's description of his work at the camp fits with the crude procedures detailed in cookbooks. He even mentions that Khabab was aware of his limitations for producing more complex chemicals like nerve agents. Dean also mentions that he helped Khabab develop an ICD, known as the Al Mubtakkar, that solved the problem of mixing the cyanide salt and acid just before launching the attack.

Basically, the Al Mubtakkar device works as a crude binary munition (a binary chemical munition is one in which chemical substances held in separate containers react when mixed or combined as a result of being fired, launched, or otherwise initiated to produce a CW) (Pita, 2015). It produces hydrogen cyanide when a barrier that separates the cyanide salt (potassium cyanide) and the acid (hydrochloric acid) is broken. Potassium permanganate is also included for the device to potentially produce a mix of hydrogen cyanide, cyanogen chloride, and chlorine. The device can be activated manually or by using the explosive triacetone triperoxide (commonly known as TATP) and a detonator. The detonator can be activated remotely or with a temporizer, allowing terrorists to escape but, if it is not well-regulated, the explosion will inactivate the chemical reagents and obviate production of the cyanogen agent.

One of the most relevant discoveries in Afghanistan regarding the chemical and biological weapons program was made by *Wall Street Journal* reporter Alan Cullison, who obtained two computers from a looter who allegedly stole them from Al Qaeda's central office in Kabul on November 12, 2001 (Cullison, 2004; Cullison and Higgins, 2001). The looter told Cullison he had found the computers in the office of Al Qaeda's military commander Muhammad Atef (aka, Abu Hafs), a strong supporter of Al Qaeda's acquisition of WMD, who was killed in a US air strike that same month. Computer files included information of Al Qaeda's effort to start a chemical and biological weapons program code-named "Al Zabadi" ("Yogurt") in May 1999 with an initial budget of only $2000−4000. Based on Cullison's analysis of the computer files, Ayman Al Zawahiri—Al Qaeda's second-in-command at that time—and Abu Hafs (assisted by Abu Khabab) started the program after studying different books and articles from biomedical journals.

According to former CIA Director George Tenet, Al Qaeda became interested in chemical and biological weapons after Aum Shinrikyo's 1995 sarin attack on the Tokyo subway (Tenet and Harlow, 2007). However, an electronic message sent by Al Zawahiri to Hafs in 1999 stated that it was "the enemy" who brought these weapons to his attention, possibly US Secretary of Defense William Cohen (Leitenberg, 2004, 2005). In November 1997, Cohen appeared on television showing a 5-pound sugar package and saying that if it were to contain spores of *B. anthracis* and were spread over Washington, DC, half the city's population would die. A photograph of Cohen holding the 5-pound sugar package was allegedly also found in Afghanistan (Leitenberg, 2004).

No public report of sophisticated CBRN means or production facilities found in Afghanistan was made. Only a centrifuge and an "oven" found near Kandahar were presented by the US Department of Defense as the equipment Al Qaeda intended to use to produce chemical and biological weapons (Miller, 2002). This material was part of a laboratory that was being built to produce *B. anthracis* spores (Pita and Gunaratna, 2009).

Based on intelligence assembled from collected documents, detainee interviews, and reconnaissance of Al Qaeda facilities during Operation Enduring Freedom, the Commission on the Intelligence Capabilities of the US Regarding Weapons of Mass Destruction concluded in its unclassified report that Al Qaeda did not have large-scale chemical and biological weapons capability (US WMD Commission, 2005). Still, past and current chemical and biological programs are said to be not fully understood, especially because of difficulties in penetrating the terrorist network and, therefore, in collecting human intelligence (HUMINT).

7.9.2.2 Daesh

Since 2014, the occupation of territories in Iraq and Syria by Daesh allowed it to exploit the production capabilities of industrial facilities and institutions working with chemicals, not only to improve the production of explosives and improvised explosive devices (IEDs) but also to develop the use of TICs as weapons. Daesh's chemical program is a continuity of Al Qaeda's program developed in Afghanistan. In fact, it is known that Abu Musab Al Zarqawi visited Abu Khabab's camp in 1999 (Dean et al., 2018). He showed interest not only in explosives but also in Khabab's developments in toxic chemicals.

There have been different reports of Daesh's use of IEDs with chlorine cylinders, and munitions filled with TICs as well as sulfur mustard in Iraq and Syria (Binder et al., 2018; Chivers, 2015; Hart, 2016, 2017; Kilic et al., 2018; Said and Lister, 2015; Sezigen et al., 2019; Strack, 2018). Moreover, the OPCW-UN Joint Investigative Mechanism (JIM) in Syria, which operated from August 2015 to November 2017, concluded that Daesh was responsible for at least two sulfur mustard attacks in Syria, one with artillery shells at Marea on August 21, 2015 (Sezigen et al., 2019; UNSC, 2016), and another with mortar rounds at Umm Hawsh on September 15−16, 2016 (UNSC, 2017).

The first reports of the use of sulfur mustard by Daesh in Iraq and Syria initially suggested that supplies from the Syrian government's chemical weapons stock had fallen into the terrorist organization's hands, or that they had even used remains from Iraq's old chemical weapons program of the 1980s that were either abandoned or had escaped Iraqi government control (Doornbos and Moussa, 2016; Entous, 2015). However, the results of the investigations of the JIM in Marea, as well as the information obtained by the OPCW in the attacks against the Kurdish peshmerga in Makhmur (Iraq), also in August 2015, suggested instead the use of low-quality sulfur mustard with a high level of polysulfides, synthesized by the so-called Levinstein procedure (UNSC, 2016).

The required chemical reagents for sulfur mustard production would be available from chemical plants, especially petrochemical plants and academic institutions, seized in the occupied territories (Gareth, 2017; Warrick, 2019). In addition, Daesh would have had access to personnel with chemical engineering knowledge, or with links to chemical weapons programs. One example is Sulayman Dawud Al Bakkar—also known as Abu Dawud—who was captured by American troops in February 2016 in the city of Tal Afar, some 60 kilometers west of Mosul (Cooper and Schmitt, 2016; Parrish, 2016; Robert, 2016). Al Bakkar was presented by the US Department of Defense as the person primarily in charge of Daesh's chemical weapons program. His experience

came from working in the former chemical weapons program of Saddam Hussein at the Iraqi Military Industrial Commission. Daesh's access to these material and human capabilities would have allowed them to start the production of sulfur mustard to load into mortar grenades, rockets, and artillery shells.

Nevertheless, the poor quality of the sulfur mustard and its use in conventional munitions, not specifically designed for the dispersion of toxic chemicals, mitigated the impact of the chemical attacks and limited the number of casualties (Dorrian, 2017). In an attempt to improve efficacy, trials were made with solid matrices, in the form of very small particles, into which the chemical agent could be absorbed to increase its persistence (Blau, 2016). Due to the limitations of the available sulfur mustard, most of the chemical attacks combined conventional ammunition with a small amount loaded with sulfur mustard. While conventional weapons were the main cause of casualties and material damage, these attacks also took advantage of the psychological effect produced by sulfur mustard which, despite its poor quality, caused blisters on the skin of those coming into contact with it.

Interrogation of captured members of Daesh involved in the chemical program allowed for the identification and bombing of many sites related to the production and storage of Daesh's sulfur mustard during the 2016 Mosul Offensive (Hart, 2016, 2017, 2018; Strack, 2018; Warrick, 2019). While Daesh's chemical capability was degraded, some small-scale attacks took place during the Battle of Mosul in late 2016 and 2017 (Browne and Starr, 2017; Hart, 2018). Although there were reports about a relocation of chemical production capabilities to the Syrian border, between the towns of Mayadin and Al Qaim in the Euphrates River Valley, most probably these attacks were made with the remains of the old production (Browne and Starr, 2017; Strack, 2018). Abandoned munitions that tested positive for sulfur mustard were found by Coalition Forces in the retaking of Mosul (Hart, 2018).

7.9.2.3 Trends

After the disappearance of the Afghan training camps and with the fall of the Daesh bastions of Mosul and Raqqa, training electronic resources have acquired more relevance in the jihadist terrorism network. Jihadist websites and social media channels are important tools providing autonomous cells and lone actors with training manuals as well as lessons learned from previous attacks (Fig. 7.2). These electronic training manuals include information and procedures about toxic chemicals like those found in training camps, that is, similar to the information included in cookbooks. Some of these websites offer scanned copies of these cookbooks.

In 2018, Europol reported that the number of online jihadist propaganda messages and tutorials proposing easy-to-implement scenarios for small-scale CW attacks had increased when compared to previous years (Europol, 2018). It also alerts to the threat of foreign terrorist fighters returning from combat zones, with the possibility that some of them may have experience with toxic chemicals which they may try to use in other countries.

7.9.3 Plots with chemical weapons

A detailed study of incidents with CWs linked to Al Qaeda shows that cyanides, ricin, and TICs have been the main choices of jihadist terrorists (Pita, 2007). Nerve agents also seem to be of interest, especially because of their toxicological and physicochemical properties, which make them ideal for tactical use in terrorist attacks. However—and as previously mentioned—the synthesis process requires some level of expertise and is far more complex than the recipes featured in jihadist manuals.

7.9.3.1 Nerve agents

Open sources have reported jihadist cells planning terrorist attacks with nerve agents and showing interest in these CWAs. In any case they had the chemical agent or proved that they had acquired the capability to produce it.

The *Daily Telegraph* (London) reported that members of an Al Qaeda cell based in the United Kingdom were arrested while planning to release sarin in the European Parliament building in Strasbourg in February 2001, although no actual evidence of sarin was found (Bamber et al., 2001). Also in February 2001, the *Daily Telegraph* reported that British police had foiled a plot involving sarin against the London underground (Hastings and Bamber, 2001). Once again there was no evidence of the actual presence of the nerve agent. Both incidents seemed to be linked and related to a transnational network of Al Qaeda cells in the United Kingdom, Germany, Italy, Belgium, France, and Spain. What these cells had in common was that their key members had been trained in Afghan camps in the mid to late 1990s. The network was planning terrorist attacks with conventional weapons and chemical substances. In a conversation on March 13, 2001, that was bugged, a member of the Italian cell mentioned "an extremely efficient liquid that suffocates people," suggesting it could be placed in tomato cans, exposing people when the cans were opened (Finn and Delaney, 2001). Referring to the German cell, he mentioned, "They arrested them while they were preparing the gas." The chemicals this cell possessed were acquired in 48 separate purchases at pharmacies but were intended to be used in IEDs, not in nerve agent production (Finn, 2002; Finn and Delaney, 2001; Pita, 2007).

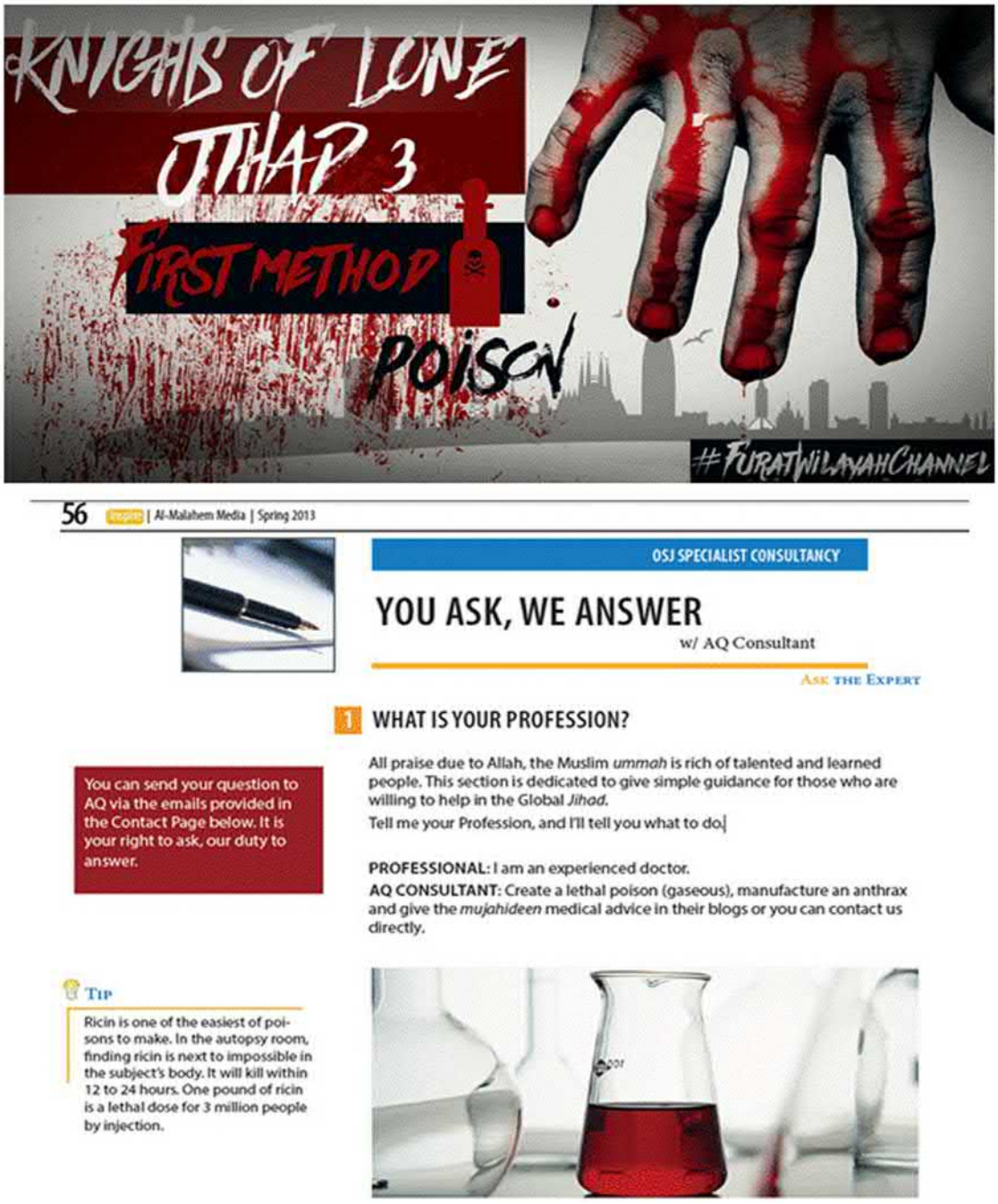

FIGURE 7.2 Jihadist electronic publications that address the use of toxic chemicals by lone actors.

On November 14, 2003, the following headline appeared in the Spanish newspaper *La Razón* (Madrid): "The FBI believes that the Salafists intended to mix napalm with sarin gas" (Arnuero, 2003). This was in reference to the chemical substances found in possession of a supposed Al Qaeda-related cell whose members were arrested in Barcelona in January 2003. However, sarin was not present among the chemicals, nor were any of its precursors. Later, it was made public that the Spanish cell, helped by other cells based in France and the United Kingdom, was planning a chemical attack against a naval base in Rota (Spain), used jointly by Spain and the United States (Martínez and Muñoz, 2005).

On September 30, 2004, the Special Advisor to the Director of Central Intelligence on Iraq's WMD reported that the Al Abud network (a network of Iraqi insurgents) attempted to produce tabun, nitrogen mustard, and ricin from late 2003 to mid-2004 (Duelfer, 2009;

Iraq Survey Group, 2004). These attempts failed, but nine mortar rounds were filled with malathion, an organophosphate insecticide.

Based on an internal British police document and on information disclosed by a UK senior officer, the *Sunday Times* (London) reported in August 2005 that Scotland Yard had thwarted an Al Qaeda attack on the House of Commons with "chemicals, a dirty bomb, and sarin gas" (Leppard and Winnett, 2005). The report mentioned that the plot was discovered after decoding encrypted e-mail messages in 2004, but there was no mention of the actual presence of the agents. This report was presumably linked to the arrest of eight men in August 2004 in the United Kingdom who were charged with conspiracy to commit public nuisance using "radioactive materials, toxic gases, chemicals, and/or explosives" (Pita, 2007). No chemicals were found, although one of the arrested men, Dhiren Barot, had two notebooks with information of explosives and toxic chemicals, as well as reconnaissance information of financial facilities in the United States. Later, Barot was reported to have been a trainer in Afghanistan camps in the late 1990s.

In June 2013, the Iraqi Defense Ministry said that they foiled a plot by an Al Qaeda cell to use remote-controlled toy planes to disseminate CWs, including sarin and sulfur mustard (Roggio, 2013). The cell was allegedly planning to execute the attacks in the Middle East, Europe, and North America. However, when the five cell members were arrested, they had not yet been able to produce any CWAs.

7.9.3.2 Cyanides

The first case of an Al Qaeda terrorist attack linked to chemical terrorism was the February 1993 World Trade Center bombing, as it is commonly believed that the explosives were mixed with a cyanide compound. The reason being that during the trial the judge stated: "You had sodium cyanide around, and I'm sure it was in the bomb. Thank God the sodium cyanide burned instead of vaporizing. If the sodium cyanide had vaporized, it is clear what would have happened is the cyanide gas would have been sucked into the north tower and everybody in the north tower would have been killed. That to my mind is exactly what was intended" (Parachini, 2000).

This was based on the idea that the terrorists considered the tactic of using a toxic chemical in the attack and the fact that the FBI found a small bottle of a sodium cyanide solution in a storage shed. However, hydrogen cyanide was never detected in the attack and even a detailed case study of this event concludes that no toxic chemical was mixed with the explosives (Parachini, 2000).

In December 1999, Jordanian authorities arrested 16 people with links to Al Qaeda who were planning attacks with explosives on New Year's Eve (Tenet and Harlow, 2007). It was later discovered that the plans also included the dissemination of hydrogen cyanide in a cinema in Amman.

On February 19, 2002, four Moroccans, members of the Salafi Group for Preaching and Combat (GSPC, now Al Qaeda in the Islamic Maghreb) were arrested in Rome (Pita, 2007). They had approximately 4 kg of potassium ferrocyanide that they intended to use to contaminate the water supplies near the US embassy. Nevertheless, this substance is widely used as a food additive (E 536) and, because of its toxicological properties, was probably not the best choice to use in a chemical attack.

Based on information in Ron Suskind's *The One Percent Doctrine* (2006), an Al Qaeda cell in Saudi Arabia planned an attack on the New York City subway with Al Mubtakkar devices in 2002. This was confirmed in 2018 by Aimen Dean, who was asked to train four cell members in Morocco on how to build the device before traveling to the United States (Dean et al., 2018). Surprisingly, Al Zawahiri decided to cancel the operation. Suskind's book claims that this decision was made just 45 days before the intended attack. One of the members of the Saudi cell had the blueprints for the Al Mubtakkar in a laptop when they were arrested in February 2003. Later, security services also found large quantities of chemicals in the desert of Riyadh, where the cell had tested the device (Dean et al., 2018). Dean was again contacted in 2004 to build Al Mubtakkar devices to attack nightlife centers frequented by US military personnel in Bahrain (Dean et al., 2018). The plot was thwarted when Dean informed the MI6 and Bahraini authorities arrested the cell members.

7.9.3.3 Ricin

Ricin was reported to have been detected in January 2003 in an apartment in the north of London where North African Al Qaeda sympathizers were living (Pita, 2007). The raid took place when the United Kingdom received a tip-off from the Algerian intelligence services indicating that "poison" had been prepared there. This ricin finding turned out to be a false positive, because subsequent analyses of samples by the British reference laboratory did not identify ricin (Leitenberg, 2005). However, 20 *Ricinus communis* seeds and a written ricin extraction procedure—copied from a cookbook and downloaded from the Internet—were found (Stenersen and Lia, 2007). The alleged plot was to use ricin on door handles (perhaps with DMSO). The fingerprints on the material and the handwriting analysis identified Kamal Bourgass, an Algerian refugee who had applied for asylum in the United Kingdom in 2000. Of all the arrested men, only Bourgass was convicted, on April 8, 2005, of conspiring

to commit a public nuisance using poisons and/or explosives. At an earlier trial in 2004, he had been convicted of killing a police officer during his capture in an apartment in Manchester on January 14, 2003.

Intelligence reports from 2010 mentioned that Al Qaeda in the Arabian Peninsula was trying to acquire large quantities of *R. communis* seeds in Yemen to produce ricin (Schmitt and Shanker, 2011). No further information about this has been made public.

Sief Allah H., a Tunisian with links to Daesh, was arrested in Cologne (Germany) on June 12, 2018, after he had prepared 84.3 mg of a ricin extract from 3150 *R. communis* seeds (Flade, 2018). Although the product found was described as "potentially lethal," results of the analyses about the content of ricin in the 84.3 mg extract have not been made public yet. The *R. communis* seeds were obtained by mail through an online store and the recipe for ricin production was an online video, which had also been found when two Egyptians were arrested in Paris (France) the previous month.

7.9.3.4 Toxic industrial chemicals

On March 30, 2004, antiterrorism police in the UK arrested eight alleged sympathizers of Al Qaeda who were supposedly planning to use osmium tetroxide against Gatwick Airport, the London subway, and other enclosed high-traffic areas, although they did not have the chemical when arrested (Baker and Kosal, 2004). Osmium tetroxide is often used as a stain in biology laboratories. One month later, Jordanian authorities announced that they had defeated an Al Qaeda plot to use explosives and large quantities of TICs such as sulfuric acid, cyanide salts, and pesticides against the US embassy in Amman, the Jordanian prime minister's office, and the headquarters of the Jordanian General Intelligence Department (Levitt and Sawyer, 2004).

From October 2006 until mid-2007, AQI suicide terrorists in Iraq detonated vehicle-borne improvised explosive devices (VBIEDs) carrying chlorine cylinders (Pita, 2012). This new tactic clearly showed that the use of TICs is an option that may yield better results than following the crude and rudimentary procedures of jihadist terrorism-related publications to produce CWs. However, the chlorine attacks in Iraq were still rudimentary in their means of delivery. In most of the attacks, casualties were not caused by chlorine exposure, but rather because of the mechanical and thermal effects of the explosion. Also, in some attacks, chlorine was not released because the low mechanical effect of the explosion was not enough to break the cylinders. Reports of Daesh's use of chlorine, chlorine-based chemicals, and phosphine, in Iraq and Syria were frequent until 2017 (Binder et al., 2018; Chivers, 2015; Hart, 2016, 2017; Said and Lister, 2015; Strack, 2018).

At the end of July 2018, two people were arrested in Australia, accused of attempting to place an IED in an aircraft at Sidney airport (Pita and Domingo, 2017; Westbrook and Barrett, 2017; Williams, 2017). The mounting of the IED, disguised as a meat mincer, was undertaken following the direct instructions of Daesh through social networks and other electronic communications media. In fact, Australian police stated that contact with the terrorist organization was established in April since the brother of one of those arrested was a high-ranking member of Daesh in Syria. This was, therefore, a "remote-controlled" operation in which the suspects even received the components of the IED by air from Turkey. Finally, the explosive device did not even make it through airport security control, apparently because of excessive baggage weight. This caused one of the arrested to leave the airport without detonating the IED.

With the failure of this attempt, the terrorist cell began another operation, also in consultation with Daesh in Syria, to manufacture an ICD. Such a device would allow the combination of two precursors for the production and dispersion of hydrogen sulfide, a toxic chemical in gaseous state at room temperature that, once inhaled, acts upon the organism via a toxicological mechanism similar to that of cyanides producing cellular asphyxiation.

7.10 Concluding remarks and future directions

Nerve agents are the most important CWs because of their high toxicity and versatility in tactical use. However, production difficulty seems to be their most important drawback. Amateur production of CWAs or extraction of toxins using cookbooks and jihadist-related manuals has commonly been overrated, leading to supposedly catastrophic consequences of terrorist attacks. In fact, CWs are frequently described as "the poor man's atomic bomb," but this description was used at the end of World War II to explain that for a state actor trying to acquire WMD capability, chemical and biological weapons would be a more feasible option than nuclear weapons, which require more complex technical resources.

An additional problem for terrorists is the need to have a reliable delivery system. Effective dissemination may be even more difficult than obtaining the agent, especially if the objective is to cause a large number of casualties. The "art" of CWs includes the research and development of special munitions that, among other things, do not inactivate the agent by the thermal effect of the explosion. Aerosolization dispersal systems are another option. Luckily, another gap in the information in jihadist-related publications and cookbooks is the often-inaccurate information regarding delivery systems.

Despite the difficulties in obtaining and effectively disseminating toxic chemicals, the evolution of CWs in Iraq and Syria suggests that jihadist terrorism is aware of the important psychological and media effects that would be produced by the mere attempt to use them. This idea can be summarized in a message that appeared in a jihadist forum linked to Daesh in June 2016: "Of course it is not easy to get anthrax or real poison, but the mere suspicion (of its presence) will provoke within the enemy an attack on their nerves, undermining their work." Therefore, it is likely that attempts to use CWs will continue.

Advances in science and technology should also be considered. Some of the drawbacks in the production of CWs and dissemination systems may disappear in the future, making re-evaluation of threat assessments necessary. The lack of adequate procedures and programs to produce CWs may explain why, until now, jihadist terrorists have not been capable of achieving an effective chemical attack outside the conflict areas of Iraq and Syria. However, they are both interested in achieving a CW capability and are actively trying to obtain it. For this reason, it should not be dismissed that options other than self-production may also be attempted, for example, resorting to sponsoring states or black-market arms trafficking networks.

The ready access to TICs in the chemical industry makes them attractive to nonstate actors. Minimizing access to "dual-use" materials by increasing security measures in academic and industrial facilities is one of the main elements addressed in different nations' strategies for countering terrorists' efforts to use WMD. The threat of insiders who work with toxic chemicals and whose integrity may be compromised by terrorist groups is one of the main concerns of intelligence services today. Insiders who decide to participate in chemical terrorism could have a variety of motivations, from ideological to financial. Advances in science and technology have also increased the number of people working with toxic "dual-use" chemicals and with the explicit and tacit knowledge that could be used for terrorist purposes.

Analysis of the chemical terrorism threat shows how important it is that medical personnel considers CWA poisoning in the differential diagnoses in cases of suspected terrorist attacks. The excellent performance of the medical personnel involved in the Novichok incidents in the United Kingdom is a good example of the importance of training in the medical aspects of CWAs.

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Chapter 8

Organophosphate nerve agents

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8.1 Introduction

It is unfortunate that the organophosphate (OP) nerve agents maintain a high profile among chemical warfare (CW) agents due to their use by terrorist groups and militant states. In the 1980s, both sarin and tabun were used by the Iraq military against Iranian soldiers (Balali-Mood and Balali-Mood, 2008). In 1988, thousands of civilians died when the Kurdish city of Halabja was attacked with sulfur mustard and nerve agents (Hay and Roberts, 1990). There were deliberate releases of the nerve agent sarin at lethal concentrations in the Japanese cities of Matsumoto (1994) and Tokyo (1995) by a Japanese domestic terrorist group. Unfortunately, the use of nerve agents for malicious intent by radical groups or by militant states continues. In 2013, sarin was deployed during an attack in Ghouta near Damascus, Syria (Dolgin, 2013; John et al., 2013; Rosman et al., 2014). Sarin was used in a 2017 attack on Khan Sheikhoun, Syria (UNSC, 2017a,b), and VX was used in the assassination of Kim Jong-nam at Kuala Lumpur International Airport in 2017. Historically, OP nerve agents were used by military authorities of several nations to develop munitions (e.g., Germany during the Nazi era, the United States, the former Soviet Union).

This chapter addresses the OP nerve agents tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), and VX. All of these are, or have been, part of the US domestic munitions inventories (Carnes, 1989; Opresko et al., 1998; NRC, 1999). Russian VX (often represented as VR) is evaluated in Chapter 10 by Rembovskiy et al. (2015). Other less well-characterized nerve agents such as compound GE, VG (Amiton), or V_x are evaluated as data allow.

The OP nerve agents are potent anticholinesterase compounds that were deliberately formulated to induce debilitating effects or death. They are notably more potent than OP pesticides. US military stockpiles of CW munitions manufactured as a Cold War deterrent decades ago are undergoing destruction at designated stockpile sites and have been the subject of extensive emergency preparedness and response planning. Additional planning has been necessary at other current and formerly used military sites where containers and buried munitions have been inventoried (the "nonstockpile" sites) (Opresko et al., 1998; NRC, 1999, 2003). More recent global events necessitate continued attention on the potential threat of chemical terrorism, especially at transportation hubs (Tucker and Raber, 2008). The Matsumoto (1994) and Tokyo (1995) incidences affirm that such attacks are a reality that require advance emergency preparedness planning (Morita et al., 1995; Okumura et al., 1996, 2007; Sidell, 1996; Cannard, 2006; Yanagisawa et al., 2006; Tu, 2007). Information provided on agent toxicity, risk assessment, treatment options, and other related topics will be useful to communities and facilities developing and updating emergency preparedness plans for accidental or intentional release of nerve agents; this information can also be used to support environmental decision making where nonstockpile material and military gear have been found. In response to various public laws and international agreements such as the Chemical Weapons Convention (PL 99−145, 1986; PL 102−484, 1993), existing emergency guidance and military policy documents currently reflect the identified criteria and information (OASA, 1999; CSEPP, 2003, 2006a,b; NRT, 2008).

Although there are different applications for the information provided in this evaluation, in general the toxicological focus for emergency preparedness and response applications is that of acute exposures associated with a one-time release. Typically, the scenarios considered include a single-source airborne release from either an intentional terrorist attack, or an accident involving an agent container or munition from a military site. It is widely recognized that vapor inhalation is the exposure route of greatest concern for such an event (Sidell et al., 1997; ATSDR, 2007). In contrast, the toxicological focus

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of environmental site remediation plans for military installations and formerly used defense sites where buried CW agent residues may occur, requires consideration of long-term release and potential incidental ingestion of media such as soil particles or water with relatively low levels of contamination. To reflect these critical applications and information needs, the current evaluation will also primarily focus on:

- single-source, one-time nerve agent releases and exposure routes involving agent vapor inhalation or direct ocular vapor exposures;
- long-term (chronic or subchronic) exposure from residual nerve agent contamination.

It is acknowledged that there exists a rich and valuable body of repeat-exposure studies using serial vapor or serial injection exposures for the nerve agents soman (GD), sarin (GB), and VX (see recent excellent reviews and analyses by Shih et al., 2006; McDonough et al., 2008; and experimental studies by Dabisch et al., 2005, 2007a). The interested reader is encouraged to examine these and related resources, because the current evaluation does not highlight experiments that apply serial exposure protocols.

8.2 Background

8.2.1 Development of organophosphate formulations as chemical warfare agents

The G-series nerve agents evaluated are all ester derivatives of phosphonic acid containing either a cyanide (GA) or a fluoride (GD, GE, GF) substituent and are commonly termed "nerve" agents as a consequence of their anticholinesterase properties as well as their effects on both the peripheral nervous system (PNS) and central nervous system (CNS). The "G" series military nomenclature used by NATO member nations has been historically considered to be an abbreviation for "German," with the second letter of the code ("A," "B," and so on) identifying the order in which these compounds were found and analytically identified by Allied forces investigating materials found in captured German military facilities at the close of WWII (Sidell et al., 1997). Alternately, the G designation is also attributed to **G**erhard Schrader, a German chemist often considered to be the "Father of Nerve Agents" in reference to his work on insecticide development and subsequent synthesis of tabun (agent GA) in 1936 (Lopez-Muñoz et al., 2008). Agent VX, a phosphonic acid ester with a sulfur substituent, was industrially synthesized in the United Kingdom in the early 1950s; the code letter "V" is a reported reference to "venom or venomous" (Sidell et al., 1997). Other, less well-characterized V-series compounds include Vx, VE, VM, and VG (trade name Amiton when commercially introduced as a miticide in the mid-1950s).

As Cold War deterrents, nerve agents began to be manufactured and weaponized by the United States in the 1950s. When the US CW agent production program was terminated by the Nixon presidential "Statement on Chemical and Biological Defense Policies" of November 1969 (National Security Decision Memorandum 35), the US stockpile of unitary munitions included bulk ("ton") containers, underwing spray tanks, projectiles, rockets, bombs, land mines, and rockets (Carnes, 1989; Sidell et al., 1997). Nerve agent unitary munitions contained GA, GB, or VX.

8.2.2 Destruction of nerve agent stockpiles

The Organization for Prohibition of Chemical Weapons (OPCW), the implementing body for the Chemical Weapons Convention (CWC), oversees the global endeavor to permanently and verifiably eliminate chemical weapons. The OPCW was awarded the 2013 Nobel Peace Prize for its extensive work in eliminating chemical weapons (OPCW, 2019a). As of April 30, 2019, 96.99% of the world's declared chemical weapons stockpiles (all chemical weapons including nerve agents) had been destroyed (OPCW, 2019b).

Consistent with this effort, US CW agent munition stockpiles have been undergoing destruction. As of 2019, the remaining stores of nerve agent in the United Sates are at Blue Grass Amy Depot, KY. Destruction of nerve agent at the Blue Grass Chemical Agent-Destruction Pilot Plant will use neutralization followed by supercritical (high temperature and pressure) water oxidation. Operations are expected to begin in 2019 with scheduled completion by 2023 (PEOACWA, 2019).

8.2.3 Physical and chemical properties of nerve agents

The G-agents are all viscous liquids of varying volatility (vapor density relative to air between 4.86 and 6.33) with faint odors ("faintly fruity" or "spicy," "odor of camphor"). Agent VX is an amber-colored liquid with a vapor density of 9.2, and is considered odorless. Thus, nerve agent vapors possess little to no olfactory warning properties (Table 8.1).

The vapor pressures and acute toxicity of these agents are sufficiently high for the vapors to be rapidly lethal. Within the G-series, GB is considered to present the greatest vapor hazard (order of vapor hazard approximates GB > GD > GF > GA). Agent VX was deliberately formulated to possess low volatility; VX is approximately 2000-times less volatile than nerve agent GB (DA, 1990a,b). As a consequence, agent VX is considered a persistent

TABLE 8.1 Physical and chemical properties of organophosphorous nerve agents.

Parameter	GA	GB	GD	GF	VX	GE	Vx
CAS registry no.	77-81-6	107-44-8	96-64-0	329-99-7	50782-69-9	1189-87-3	20820-80-8
Chemical name[a]	Ethyl dimethylamido cyanophosphate	Isopropyl methyl-phosphono-fluoridate	Pinacolyl methyl-phosphono-fluoridate	*O*-Cyclohexyl methyl-phosphono-fluoridate	*S*-(2-diisopropyl-aminoethyl) *O*-ethyl methyl phosphonothiolate	Isopropyl ethylphosphono-fluoridate	*O*-Ethyl-*S*-(2-dimethylaminoethyl) methyl-phosphonothiolate
Common name[a,b]	Tabun	Sarin	Soman	Cyclosarin	VX	NA	NA
Chemical formula[a]	$C_5H_{11}N_2O_2P$	$C_4H_{10}FO_2P$	$C_7H_{16}FO_2P$	$C_7H_{14}FO_2P$	$C_{11}H_{26}\ NO_2PS$	$C_5H_{12}FO_2P$	$C_7H_{18}NO_2PS$
Molecular weight[a]	162.13	140.10	182.178	180.2	267.38	154.12 (calculated)	211.26
Physical state[a,c]	Liquid, vapor	Liquid, vapor	Liquid, vapor	Liquid, vapor	Oily liquid, vapor	Vapor	Liquid
Vapor pressure (mmHg)[a]	0.037 (20°C)	2.10 (20°C)	0.40 (25°C)	0.056 (20°C)	0.0007 (25°C)	NA	6.73×10^{-3} (25°C)
Volatility (mg/m^3 at 25°C)[a,c]	610	22,000	3900	548 at 20°C; 817 at 25°C	10.5	11.6 mg/L at 25°C (saturated concentration)	76.4
Liquid density (g/mL)[a]	1.073 (25°C)	1.102 (20°C)	1.0222 (25°C)	1.1327 (20°C)	1.006 (20°C)	1.0552 (25°C)	1.06 (25°C)
Vapor density (air = 1)[a]	5.63	4.86	6.33	6.2	9.2	NA	7.3 (calculated)
Melting point (°C)[a,b,c]	−50	−56	−42	−30	−39 (calculated)	NA	NA
Boiling point (°C)[a,b,c]	245	158, 150	198	239	298	67–68	256 (extrapolated)
Water solubility[a,c]	98 g/L (25°C); 72 g/L (20°C)	Miscible	21 g/L (20°C)	0.37% (20°C)	30 g per 100 g (25°C)	NA	Slightly
Hydrolysis half-life[d] (20°C and pH 7)	8.5 h	39–41 h; 80 h	80–83 h; 45 h at pH 6.65	42 ha	400–1000 h	NA	NA

(*Continued*)

TABLE 8.1 (Continued)

Parameter	GA	GB	GD	GF	VX	GE	Vx
Log K_{ow}[e]	1.18	0.15	1.02	NA	NA	NA	NA
Odor[a,b,c,f]	Faintly fruity; no odor when pure	Odorless when pure	Fruity, odor of camphor when impure	Perceptible; fruity; no agreement on odor description; odorless when pure	Odorless when pure	NA	Odorless
Odor threshold (mg/m^3)[a,b,c,f]	Undefined	<1.5	~1.5 to ~7.0	~10.4 to ~14.8	Odorless when pure	Undefined	Undefined
Henry's law constant[e,g] (atm m^3/mol)	1.52×10^{-7}	5.34×10^{-7}	4.56×10^{-6}	NA	3.5×10^{-9} (est.)	NA	NA
Conversion factors[h] in air	ppm = (0.15) × mg/m^3 mg/ m^3 = (6.6) × ppm	ppm = (0.17) × mg/m^3 mg/ m^3 = (5.7) × ppm	ppm = (0.13) × mg/m^3 mg/ m^3 = (7.5) × ppm	ppm = (0.14) × mg/m^3 mg/m^3 = (7.4) × ppm	mg/m^3 = (10.936) × ppm ppm = (0.0914) × mg/m^3	NA	NA

[a] *Gates and Renshaw (1946); Buckles (1947); DA (1990a,b); Abercrombie (2003); Tevault et al. (2003).*
[b] *DA (1992).*
[c] *DA (1974); Yang (1999).*
[d] *Clark (1989); DA (2005).*
[e] *Britton and Grant (1988).*
[f] *Dutreau et al. (1950); McGrath et al. (1953); DA (2005).*
[g] *Small (1984); Opresko et al. (1998).*
[h] *Calculated from molecular weight.*
Source: Adapted from National Research Council (NRC), 2003. Committee on Toxicology, Board on Environmental Studies and Toxicology, Commission on Life Sciences. The National Academies Press, Washington, DC, with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington, DC.

"terrain denial" military compound with the potential to be a contact hazard or generate off-gas toxic vapor concentrations over a period of days after surface application, particularly under cold weather conditions or when bulk-release quantities of liquid agent are involved. Although not readily volatile, VX vapors (if allowed to accumulate) are nevertheless considered more acutely potent than those of agent GB or the other G-series agents (Mioduszewski et al., 1998).

As a consequence of the volatilities exhibited by G-series nerve agents (Table 8.1), the most likely exposure route (and source of primary hazard) is via direct vapor exposure to the eyes and upper respiratory tract tissues and vapor inhalation (with consequent systemic absorption) (Cannard, 2006; Dabisch et al., 2008a); G-agents are considered "nonpersistent" as per definitions used by the US Department of Defense (DOD, 2008). Nerve agent VX is widely considered to present a greater threat from the percutaneous exposure route (when compared with the G-series agents) as well as a vapor inhalation threat at elevated ambient temperatures (e.g., >40°C) (Craig et al., 1977; Sidell et al., 1997; Benton et al., 2005, 2006a).

Nerve agent V_x exhibits volatility (76.4 mg/m^3 at 25°C) intermediate to that of agents GA and VX, and a vapor density (7.3) intermediate to that of agents GF and VX; V_x is also considered "persistent." There are few data from which to characterize nerve agents VE (*O*-ethyl-*S*-[2-(diethylamino) ethyl]ethylphosphonothioate, CAS No. 21738-25-0) or VM (*O*-ethyl-*S*-[2-(diethylamino)ethyl]methylphosphonothioate, CAS No 21770-86-5).

8.2.4 Mode of action and clinical signs

All of the OP nerve agents under consideration are cholinesterase inhibitors that phosphorylate acetylcholinesterase (AChE), thereby preventing the inactivation of the neurotransmitter acetylcholine (ACh) at neural synapses and neuromuscular junctions (NMJs). Depending on the route of exposure and amount absorbed, the PNS and/or CNS can be affected. The result is prolonged stimulation of muscarinic and/or nicotinic receptors. The mnemonic, SLUDGE, refers to muscarinic-mediated effects of *S*alivation, *L*acrimation, *U*rination, *D*efecation, *G*astrointestinal distress, and *E*mesis. An abbreviation (MTWTF) referring to days of the week refers to nicotinic receptor-mediated responses of *M*iosis, *T*achycardia, *W*eakness, Hypertension/Hyperglycemia, and *F*asciculations. OP nerve agent interaction with other esterases may also occur, and direct effects to the nervous system have been reported.

In addition to the previously noted array of effects and mediated by similar mechanisms, exposure to acutely toxic concentrations of nerve agents may cause paralysis, loss of consciousness, convulsions, depression of respiratory centers, and death (Grob and Harvey, 1953; Grob, 1956; Sidell et al., 1997; Yanagisawa et al., 2006; Marrs et al., 2007a; Okumura et al., 2015). Minimal effects observed at low vapor concentrations include miosis (contraction of the pupils of the eye, with a subsequent decrease in pupil area), tightness of the chest, rhinorrhea, and dyspnea (Dunn and Sidell, 1989; Dunn et al., 1997). Pupillary contraction, resulting in varying degrees of miosis characterized by measures of pupil diameter, is consequent to local inhibition of ocular AChE activity with pupillary sphincter contraction (Dabisch et al., 2007b, 2008a,b; Dabisch et al., 2010).

Using social media (YouTube), Rosman et al. (2014) provided a unique and informative analysis of the Damascus area attack in 3013 that killed 1400 civilians in Syria. The observations were consistent with signs of toxicity consistent with OP nerve agent exposure. An objective analysis by physicians who were chemical, biological, radiological, and nuclear specialists analyzed 67 videos that met various inclusion criteria. These videos showed 130 casualties, 119 of which were evaluated as having moderate injuries or worse. Clinical signs of dyspnea, diaphoresis, and loss of consciousness were observed in 53%, 48.4%, and 40.8%, respectively, of the casualties. Miosis was observed in 13.8% of victims, hypersalivation in 32.3%, and convulsions in 18.5% of victims.

Reactivation of inhibited cholinesterase by dephosphorylation is not possible once the nerve agent−cholinesterase complex undergoes "aging," which is thought to be the consequence of the loss of an alkyl or alkoxy group. Agent GD ages very rapidly when bound to red blood cell cholinesterase (RBC−ChE), with a $t_{½}$ (time required for 50% of the enzyme to become resistant to reactivation) of 1.3 min (Harris et al., 1978). The aging half-time for agent GA with RBC−ChE is 46 h (calculated; De Jong and Wolring, 1978), and the $t_{½}$ for agent GB with RBC−ChE is 5 h (Sidell and Groff, 1974). The complex formed between RBC−ChE and agent VX does not age significantly (half-life of approximately 48 h) (Sidell and Groff, 1974; Dunn et al., 1997).

8.2.5 Direct nervous system effects

Nerve agents exert toxic effects on the CNS and PNS indirectly through AChE inhibition (Koelle, 1981; Koelle et al., 1975), but may also affect nerve impulse transmission by additional mechanisms at NMJs (see reviews by Somani et al., 2001; Marrs et al., 2007a; Gupta et al., 2010) and at neurotransmitter receptor sites in the CNS (Myhrer et al., 2010; Weissman et al., 2010). Rao et al. (1987) reported that VX caused an increase in ACh release at NMJs in the frog by an interaction with the nicotinic ACh receptor−ion channel complex. Aas et al. (1987) reported alterations in muscarinic receptors in rat

bronchi and lung tissue after subacute inhalation exposures to agent GD. In the CNS, nerve agents may act directly on muscarinic, nicotinic, and glutamate receptors in manners unrelated to cholinesterase inhibition (Bakry et al., 1988; Chebabo et al., 1999; Lallement et al., 1991a, b; Rocha et al., 1998, 1999). Chebabo et al. (1999) reported that 0.3−1 nM of agent GB reduced the amplitude of GABA-mediated postsynaptic currents (GABA; neurotransmitter γ-aminobutyric acid), but had no effect on the amplitude of glutamatergic-mediated postsynaptic currents; this selective reduction in action potential-dependent release of GABA might account for GB-induced seizures. Lallement et al. (1991a,b) had suggested that GD-induced overstimulation of glutamatergic receptors contributed to maintenance of seizures.

Although these electrophysiological data indicate that nerve agents may have direct effects on the nervous system unrelated to AChE inhibition, the data do not provide a means of determining a dose conversion to an integrative whole-body endpoint such as lethality or qualitative/quantitative comparisons directly relevant to adverse effects.

It should be further noted that the effects of nerve agents on GABAergic transmission in the CNS may have implications for behavioral effects in laboratory animals and humans, and may also contribute to the induction of convulsions at higher doses (Bakshi et al., 2000). Nevertheless, given the present undefined application of noncholinergic data to whole-body estimations, reliance on the primary assumption of AChE action is consistent with recognized opinion (Bakshi et al., 2000).

8.2.6 Binding with blood cholinesterases

The activity levels of RBC−ChE, and plasma cholinesterase (plasma−ChE, plasma butyrylcholinesterase or BuChE), have been used to monitor exposure severity as well as recovery from anticholinesterase pesticides and OP nerve agents. There is some historical evidence that RBC−ChE can be as sensitive as brain−ChE to the anticholinesterase effects of nerve agents. Grob and Harvey (1958) reported that in vitro concentrations producing 50% activity depression of brain−ChE and RBC−ChE were equivalent in the case of GA (1.5×10^{-8} mol/L), and comparable in the case of GB (3.0×10^{-9} vs 3.3×10^{-9} mol/L). The in vivo animal studies conducted by Jimmerson et al. (1989) disagree, which is further supported by the fact that blood ChE activity may not always be correlated with exposure or with signs and symptoms of toxicity (Holmstedt, 1959; Sidell and Somani, 1992; Sidell et al., 1997) (Table 8.2). This was also observed during clinical treatment of cases after the Matsumoto and Tokyo chemical terrorist incidents of GB exposure to the public (Nozaki et al., 1997; Yanagisawa et al., 2006).

It is generally considered that systemic effects in humans after acute nerve agent exposures are likely when RBC−ChE is inhibited by 75%−80% (e.g., to 20%−25% of normal activity levels) (Sidell and Somani, 1992). Nevertheless, it is well-known that local signs and symptoms of the eyes and nose in humans and animals (e.g., miosis, rhinorrhea) can occur in the absence of any measurable change from baseline ChE activity in the blood after vapor or aerosol nerve agent exposure (Harvey, 1952; Craig and Woodson, 1959; Sidell and Somani, 1992) and are attributable to the local and direct effects of the agent on tissues of the eyes and upper respiratory tract (Grob, 1956; Dabisch et al., 2008a) (Table 8.2). When systemic exposure (e.g., other than direct ocular or direct nasal) occurs, miosis and rhinorrhea are not usually observed as the first noticeable effects (NRC, 2003; Dabisch et al., 2008a).

EPA science policy guidelines regarding the use and application of cholinesterase activity inhibition data generally consider blood ChE activity inhibition to be an imperfect measure, and there appears to be no fixed percentage of blood ChE activity change that can distinguish adverse from nonadverse effects (Storm et al., 2000; USEPA, 2000). A number of investigations have noted the poor association between blood (RBC and plasma) cholinesterase activity and nerve agent intoxication (Rubin and Goldberg, 1957; Sidell and Somani, 1992; Sidell et al., 1997; Koelle, 1994; Mioduszewski et al., 2002a; Cannard, 2006; Yanagisawa et al., 2006); minimal blood ChE activity has been observed in association with normal tissue function (Sidell and Somani, 1992). In a clinical situation, measurement of blood ChE activity has forensic utility and is helpful as a measure of recovery but is not a quantitative measure of absorbed dose (Cannard, 2006).

8.2.7 Binding with other enzymes

Nerve agents also interact with detoxification enzymes such as carboxylesterases (CarbE) and A-esterases (e.g., arylesterase and paraoxonase), and the degree of such interaction can alter the magnitude and extent of the toxic cascade after AChE inhibition (Gupta et al., 1991; Pope, 1999; Pope and Liu, 2002; Fonnum et al., 2006) as well as species-specific characteristics. Observed spontaneous reactivation of soman-inhibited plasma CarbE in the rat indicates that "aging" does not occur for the GD−plasma CarbE complex (in contrast to that observed for GD and RBC−ChE) (Dunn et al., 1997), and further suggests that endogenous plasma CarbE may be a principal functional scavenger for agent GD (Maxwell and Brecht, 2001). Recent studies indicate that full characterization of the OP-protective capabilities of CarbEs requires assessment not only of the amount, but also of the affinity exhibited

TABLE 8.2 Human experimental data for single exposures to GB vapor.

Concentration (mg/m^3)	Exposure duration	Ct ($mg\ min/m^3$)	Signs and symptoms	References
0.05	20 min	1	Headache, eye pain, rhinorrhea, tightness in chest, cramps, nausea, malaise	Harvey (1952)
0.05	20 min	1	Threshold (<1 mm pupil diameter decrease) to mild (1–2 mm pupil diameter decrease) miosis[a] in test subjects	Johns (1952)
0.06	20 min	1.2	No reported effects	McKee and Woolcott (1949)
0.06	40 min	2.4	Miosis; slight tightness in chest ($n = 4$)	McKee and Woolcott (1949)
0.3	0.5 min	0.15	Rhinorrhea in 16/16; chest tightness in 7/16	Fairley and Mumford (1948)
0.5	30 min	15.0	Miosis, dyspnea, photophobia, 40% inhibition of RBC–ChE, subclinical SFEMG[b] changes	Baker and Sedgwick (1996)
0.6	1 min	0.6	Miosis and slight tightness in chest	McKee and Woolcott (1949)
2	2 min	4	Miosis "moderate"; no other signs of ChE inhibition	Rubin et al. (1957)
NA	10 min to 5 h	3.13	50% pupil area decrement	Callaway and Dirnhuber (1971)
NA	10 min to 5 h	13.85	90% pupil area decrement	Callaway and Dirnhuber (1971)
4.19 (average)	2 min	8.38	Average 47% inhibition of RBC–ChE; no other effects (breathing rate 5.6–8.4 L/min through nose or mouthpiece)	Oberst et al. (1968)
20.7 (average)	2 min	41.4	Average 49% inhibition of RBC–ChE; no other effects (breathing rate 47–65 L/min through nose or mouthpiece)	Oberst et al. (1968)
2.8–4.3	1–2.25 min	4.5–5.0	Miosis of unprotected (unbandaged)[c] eyes of 10 military servicemen; min pupil size of 1.8 mm	Sim (1956)
4.0–4.5	2–2.25 min	8.3–9.8	Miosis of unprotected (unbandaged)[c] eyes of 22 military servicemen; min pupil size of 1.6 mm	Sim (1956)
9.5	1 min, 3 s	10	Miosis of unprotected (unbandaged)[c] eyes of 12 military servicemen; min pupil size of 1.7 mm	Sim (1956)
5.5–7.6	1.75–2.5 min	13.1–15.4	Miosis of unprotected (unbandaged)[c] eyes of 54 military servicemen; min pupil size of 1.5 mm	Sim (1956)
12.8–15.3	1–1.2 min	14.4–15.0	Miosis of unprotected (unbandaged)[c] eyes of 38 military servicemen; min pupil size of 1.5 mm	Sim (1956)

[a] *Mild miosis defined by Johns (1952) as "decrease of 1–2 mm" in pupil diameter; reversible within 24 h.*
[b] *Single-fiber electromyography (SFEMG).*
[c] *Note that a similar experimental exposure protocol employed by Sim (1956) for subjects with bandaged eyes ("protected") resulted in no clinical miosis in any subject.*
Source: Adapted from National Research Council (NRC), 2003. Committee on Toxicology, Board on Environmental Studies and Toxicology, Commission on Life Sciences. The National Academies Press, Washington, DC with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington, DC.

by CarbEs for the inhibitor, as well as the total CarbE activity unlikely to be inhibited (inhibitor-resistant esterase activity) (Chanda et al., 2002). The detoxification potential of CarbEs is multifaceted and is an area requiring further experimental characterization (Fonnum et al., 2006, 2015).

8.3 Toxicity

8.3.1 Effects

Nerve agents are toxic anticholinesterase compounds by all routes of exposure and exhibit a steep dose−response curve. Detailed descriptions of nerve agent toxicity may be found in reviews by Munro et al. (1994), Sidell et al. (1997), Mioduszewski et al. (1998), Opresko et al. (1998), NRC (1999, 2003), Bakshi et al. (2000), Somani et al. (2001), Marrs et al. (2007a), Weissman et al. (2010), and Hulet et al. (2019). The toxic responses to nerve agents generally involve inhalation of and direct contact (especially for ocular effects) with vapors and aerosols. Especially with regard to aerosol exposures, it is also possible that some ingestion of the agents will occur that will result in direct effects on the gastrointestinal tract. The following sections provide an overview of key studies on OP nerve agent toxicity but are not intended to be an exhaustive encyclopedic review of the subject.

Anticholinesterase effects of nerve agent exposure can be characterized as muscarinic, nicotinic, or CNS. Muscarinic effects occur in the parasympathetic system and, depending on the amount absorbed, can be expressed as conjunctival congestion, miosis, ciliary spasm, nasal discharge, increased bronchial secretion, bronchoconstriction, anorexia, emesis, abdominal cramps, sweating, diarrhea, salivation, bradycardia, and hypotension. Nicotinic effects are those that occur in the somatic (skeletal/motor) and sympathetic systems, and can be expressed as muscle fasciculations and paralysis. CNS effects may be manifested as confusion, reflex loss, anxiety, slurred speech, irritability, forgetfulness, depression, impaired judgment, fatigue, insomnia, depression of central respiratory control, and death (Sidell and Groff, 1974; Sidell and Somani, 1992; Sidell et al., 1997; Opresko et al., 1998; Bakshi et al., 2000). Minimal effects observed at low concentrations in human subjects include miosis, a feeling of "tightness" in the chest, rhinorrhea, and dyspnea (Dunn and Sidell, 1989) (Table 8.2).

RBC−ChE inhibition in the blood is considered an operationally acceptable surrogate for CNS inhibition. Plasma ChE is more labile and is a less reliable reflection of enzyme activity change at neuro-effector sites (Young et al., 1999; USEPA, 2000). Assays to assess nerve agent exposure are continually being improved. Mathews et al. (2017) reported on a high-confidence mass spectrometry assay for OP nerve agent adducts to human butyrylcholinesterase. A variety of assays are available for assessing cholinesterase activity and have been summarized in Benedict et al. (2019).

In the whole-body agent vapor exposure studies of Mioduszewski et al. (2002a; SD rat single exposures to GB vapor) and Benton et al. (2006a; SD rat single exposures to VX vapor), miosis was usually not correlated with or accompanied by reductions of circulating AChE, BuChE, or CarbE. For the VX vapor exposure study of Benton et al. (2006a), and among those rats exhibiting only one sign (either whole-blood AChE activity inhibition or miosis), miosis developed in the absence of blood AChE activity depression "90% of the time." The findings of Mioduszewski et al. (2002a) for SD rats are consistent with those for human volunteers exposed to GB vapor in the study by Rubin and Goldberg (1957). These results further document the fact that miosis alone, and in the absence of signs such as ChE or CarbE activity inhibition, is a local effect and reflects an exposure much less than that required for the generation of systemic clinical effects. Thus, consideration of a local effect such as miosis as a critical endpoint for decision criteria and exposure guideline determination allows a useful margin of protection against the potential for agent exposures sufficiently large to generate systemic effects.

8.3.2 Minimal potential for delayed neuropathy

A continuing area of public concern regarding nerve agent exposure is the possibility of chronic neurological effects, particularly delayed neuropathy, given that neuropathic effects have been observed after high levels of occupational exposure to the lipophilic agricultural pesticides. Exposure to some OP anticholinesterase compounds results in delayed neurotoxic effects (ataxia, distal neuropathy, paralysis), which are collectively described as organophosphate ester-induced delayed neuropathy (OPIDN). OPIDN is characterized by myelin sheath and axon degeneration and was once thought to be the consequence of inhibition and aging of neuropathy (or neurotoxic) target esterase (NTE) (Abou-Donia, 1993; Ehrich and Jortner, 2002; Gupta et al., 2012). With greater knowledge and recent data pointing out that NTE-knockout mice may also develop OPIDN (Abou-Donia, 2003; O'Callahan, 2003; Winrow et al., 2003), the NTE theory has been replaced with one involving a noncholinergic, proteolytic mechanism involving cytoskeletal proteins found in neurofilaments (De Wolff et al., 2002). The resulting proteolysis, accompanied by perturbed ionic gradients, cellular edema, and myelin debris, can generate neuropathy.

A number of well-conducted studies using USEPA guidelines for experimental determination of delayed neurotoxicity (USEPA, 1998) have been performed for the G-agents and agent VX (Gordon et al., 1983; Willems et al., 1984; Goldman et al., 1988; Wilson et al., 1988). The USEPA protocol requires toxicological testing with the domestic hen, an OPIDN-sensitive laboratory animal. In general, exposure to the standard threat nerve agents (e.g., GA, GB, GD, GF, VX) is not considered neuropathic in

humans (Marrs et al., 2007a) given that agent VX is not neuropathic in standard challenge tests with hens and that G-agent concentrations necessary to induce OPIDN would be supralethal, and human survival would be highly unlikely.

8.3.3 Long-term effects following exposure to nerve agents

Although delayed OPIDN has not been demonstrated for the organophosphate nerve agents, assessments of casualties from recent civilian subversive activity and military attacks have provided evidence for long-term neuropsychological and neurobehavioral effects. Analyses of these events lack exposure terms which prevents an accurate determination of an exposure concentration−effect relationship. Exposures are typically defined by the severity of initial effects and the level of medical intervention required for immediate treatment. In a systematic review of studies addressing the long-term neurological effects of acute exposure to sarin, the National Toxicology Program concluded with a moderate level of confidence that acute exposure of humans to sarin is suspected of having long-term (≥1 year after exposure) adverse effects on learning and memory. Further, a cross-sectional study and one case report of Tokyo subway attack victims noted evidence for morphological and histopathological changes in the nervous system in the years following exposure to sarin (NTP, 2019). The report also stated that additional and more refined analyses should address areas of low confidence and inconsistencies in topics of concern such as visual and ocular effects, and long-term effects on cholinesterase. Talibani et al. (2018) reported a wide range of long-term neurological sequelae in about one-third of individuals in a study group who had been exposed during the attack on the Kurdish city of Halabja. Long-term effects of exposure to OP nerve agents have been reviewed by Figueiredo et al. (2018) and are discussed in greater detail in other chapters in this volume.

8.3.4 Evaluation of other potential effects

Animal data from vapor, oral, and injection exposure studies for the G-series nerve agents and agent VX indicate that these agents do not induce reproductive or developmental effects in mammals (Van Kampen et al., 1970; Denk, 1975; Schreider et al., 1984, 1988; Laborde and Bates, 1986; Goldman et al., 1988; Bates et al., 1990; Bucci et al., 1993; LaBorde et al., 1996). Incidental data from the Tokyo subway incident (Ohbu et al., 1997) documenting the birth of healthy children to women who had received exposures to toxic GB concentrations at 9−36 weeks of gestation support this finding.

Neither agent GB nor agent VX was genotoxic in a series of microbial and mammalian assays (Crook et al., 1983; Goldman et al., 1987, 1988), whereas agent GA has been reported to be weakly mutagenic in similar cellular assays (Wilson et al., 1994). Experimental results indicate that agents GB, GA, and VX have no carcinogenic potential (Weimer et al., 1979; Goldman et al., 1988; Bucci et al., 1992a,b).

8.3.5 Inhalation/ocular toxicity in controlled experiments with human subjects

It is noted that the most complete experimental data set for the nerve agents evaluated in all species is that for agent GB. The following analysis reflects that emphasis (Table 8.2). Human study reports evaluated have been previously judged by the US Environmental Protection Agency National Advisory Committee for Acute Exposure Guideline Levels for Hazardous Substances and the National Research Council (NRC) Committee on Toxicology to be consistent with acceptable criteria and procedures regarding informed consent and appropriate clinical supervision (NRC, 2001, 2003).

8.3.5.1 Agent GB

Fairley and Mumford (1948) exposed 16 male volunteers to 0.3 mg GB/m^3 for 0.5 min. Nine of the test subjects reported that they could detect the agent by smell, seven reported tightness of the chest and 16 reported rhinorrhea. McKee and Woolcott (1949) evaluated the effects of low concentrations of agent GB on 14 male volunteers. A single exposure to 0.6 mg GB/m^3 for 1 min or 0.06 mg GB/m^3 for 40 min resulted in miosis and slight tightness of the chest; within 24 h, signs and symptoms resolved in subjects exposed for 1 min, whereas more than 48 h was required for resolution in subjects exposed for 40 min.

In a study reported by Harvey (1952), 128 adult male volunteers were exposed in a chamber to GB concentrations ranging from 0.05 to 3.0 mg/m^3 for 2−20 min. The corresponding cumulative exposures ranged from 1.0 to 6.0 mg min/m^3. The most common signs and symptoms resulting from the GB exposures were headaches, eye pain, rhinorrhea, tightness in the chest, cramps, nausea, and concentration difficulties.

When evaluating data from the Harvey (1952) study, Johns (1952) reported on the occurrence of miosis in exposed individuals. Regression analysis of 150 observations, including 55 controls, indicated that the concentration at which a 50% decrease in pupil diameter would be attained was approximately 4.1 mg min/m^3, with 90% confidence limits of approximately 2.7 and 5.7 mg min/m^3. Johns (1952) defined “mild miosis” as a “decrease of 1−2 mm” in pupil diameter, which usually disappeared

within 24 h. Although mild miosis as defined was observed in some subjects at the lowest Ct tested ($Ct = 1.0$ mg min/m^3), other subjects exhibited mean maximal pupil decreases of less than 1 mm, indicating attainment of a response threshold at this level of exposure. Untreated controls exhibited a pupil diameter decrease of 0.33 mm or more; Johns (1952) attributed this difference to observer bias and pointed out that there was still a relative difference between the control group and the exposed groups.

Oberst et al. (1968) conducted inhalation studies in which 125 volunteers were exposed to low concentrations of GB vapor to measure levels of GB retention and changes in RBC−ChE activity. In one series of tests in which resting subjects were exposed to GB for 2 min, the calculated retained dose was 3.4−3.8 μg/kg and the percent inhibition of RBC−ChE activity was 39%−63% (average 49%). In a second series of tests in which exercising men were exposed to GB for 2 min, the calculated retained dose was 3.2−4.0 μg/kg and the percent inhibition of RBC−ChE activity was 29%−58% (average 47%). The reported 2 min ChE_{50} dose for all 125 subjects (grouped data) was 3.95 μg GB/kg. From these data, the 2 min EC_{50} for cholinesterase inhibition can be estimated as approximately 21 mg/m^3 for resting men breathing approximately 7 L/min and approximately 4 mg/m^3 for exercising men breathing approximately 50 L/min.

Baker and Sedgwick (1996) exposed eight human volunteers to 0.5 mg GB/m^3 for 30 min in a chamber; test subjects walked at a rate of 96 paces per minute while breathing normally. The exposure resulted in a 60% inhibition of RBC−AChE activity; subjects exhibited miosis, some photophobia, and mild dyspnea. Respiratory symptoms resolved within minutes and the ocular effects within 48 h after exposure. There were no clinical neuromuscular signs or symptoms; however, small and nonclinical changes in single-fiber electromyography (SFEMG) of the forearm were measured at 3 h and 3 days after exposure; SFEMG changes were not detectible 15−30 months after exposure.

The results of agent GB vapor exposure studies conducted with human volunteers indicate that the threshold for miosis and other minimal toxic effects is in the range of 0.05−0.5 mg/m^3 for 10−30 min exposures (Table 8.2 and summaries). Rubin et al. (1957) evaluated the effects of agent GB on the visual threshold of three adult volunteers. The test individuals were exposed to 2 mg GB/m^3 for 2 min with the eyes exposed or protected. With the eyes unprotected, the exposure resulted in moderate miosis with no other obvious signs of cholinesterase activity inhibition, but with a significant elevation of the absolute visual threshold in the dark-adapted eye.

Callaway and Dirnhuber (1971) evaluated the "miotogenic potency" of GB vapor in humans (62 miosis responses in 26 human volunteers). Exposure time periods ranged from 10 min to 5 h. Callaway and Dirnhuber reported 50% and 90% decrements in pupil area (Table 8.2). There are acknowledged weaknesses in the protocol and data of Callaway and Dirnhuber (1971), such as limited 1970s-era capabilities for measuring agent vapor concentrations, semisubjective protocols for measuring miosis in human eyes, and incomplete documentation of miosis incidence.

Based on human and animal data, McNamara and Leitnaker (1971) estimated that the EC_{50} for miosis in humans would be 0.0083 mg/m^3 for 8 h exposure duration or 0.0028 mg/m^3 for 24 h exposure duration. McNamara and Leitnaker (1971) did not expect miosis to occur at 0.001 mg/m^3 for 8 h or 0.0003 mg/m^3 for 24 h.

8.3.5.2 Agents VX and Vx

No experimental data are available for direct characterization of acute VX vapor toxicity in humans after inhalation exposure. Based on lethality data for several animal species, Bide and Risk (2000, 2004) estimated the 10 min LCt_{50} value for a VX aerosol to be 7 mg min/m^3 for a 70-kg man breathing 15 L/min for 10 min.

One of the few experimental attempts to evaluate human exposure to VX vapor for durations longer than a few minutes is the historically important study of Bramwell et al. (1963), in which eight individuals were exposed to VX vapor concentrations ranging from 0.23 mg/m^3 to 5 mg VX/m^3 for durations ranging from 2.25 s to 24 min ($Cts = 0.7−25.6$ mg min/m^3). The Bramwell et al. (1963) study is not considered credible because of its seriously flawed exposure protocol; both C and t were varied (resulting in no replicate cumulative exposures), and the organic solvent benzene was used to help disperse the agent in the exposure (carrier solvent may have altered agent absorption) (Reutter et al., 2000).

Koon et al. (1959) evaluated the minimum odor detection limits of VX in 16 volunteers. Each subject sniffed the agent both during the morning and afternoon on 2 successive days (presumably only one sniff at each time point). The estimated total doses for the four exposures ranged from 0.01 to 0.13 μg/kg. No significant changes in RBC or plasma ChE activity were observed in the test subjects. Three subjects reported headaches the evening of the last test, and three other subjects reported slight chest tightness, dryness of the mouth, and nasal irritation for 30 min after the test.

Recent multiservice (Army, Marine Corps, Navy, and Air Force) guidance on agent-specific exposure limits estimates the VX ECt_{50} for mild toxicity in humans (miosis, rhinorrhea) to be 0.10 mg VX-min/m^3 for 2−360 min exposures (DA, 2005). The inhalation/ocular ECt_{50} for severe effects in humans (i.e., muscular weakness,

tremors, breathing difficulty, convulsions, paralysis) was estimated to be 10 mg min/m^3 for 2−360 min exposures for a respiratory minute volume of 15 L/min (DA, 2005).

Agent Vx is considered toxic via inhalation exposure or direct contact with the eye and/or skin (DA, 2005) but has been poorly studied. Because of lack of data suitable for analysis, DA (2005) has determined that no toxicity estimates for Vx can be developed at this time.

8.3.6 Inhalation/ocular toxicity in laboratory species

8.3.6.1 G-series agents

8.3.6.1.1 Lethal levels

Data regarding the acute lethality of G-series agents for short-term exposures are summarized in Table 8.3 (see also NRC, 2003, for a detailed review). In studies conducted by Mioduszewski et al. (2001, 2002a), acute lethality of agent GB to male and female SD rats was evaluated for time periods of 10, 30, 60, 90, 240, and 360 min in a whole-body dynamic chamber. GB concentrations ranged from approximately 2 to 56 mg/m^3, and lethality was assessed at 24 h and at 14 days after exposure. Female rats were reported to be significantly ($P < .01$) more sensitive than males to GB vapor toxicity over the range of exposure concentrations and durations studied.

In studies conducted by Bide and Risk (2004), male CD1 strain mice were exposed to whole-body GB for time periods ranging from 20 to 720 min. LC_{50} values for 3−12 h were progressively higher (toxicity lower) than that predicted by either Haber's rule or the Ten Berge relationship (Ten Berge et al., 1986). In studies conducted by Anthony et al. (2004), male and female SD rats were exposed to whole-body agent GF for 10, 60, or 240 min, and lethality was assessed 24 h and 14 days after exposure (Table 8.3). Females were more sensitive than males.

Hulet et al. (2006b) exposed male and female Göttingen minipigs to whole-body lethal concentrations of agent GF vapor for 10, 60, or 180 min (Table 8.3). No significant gender differences were observed in the GF lethality values.

In the latter years of WWII, agent GE underwent acute inhalation toxicity characterization at a number of research facilities managed by the Office of Scientific Research and Development (National Defense Research Committee). These results, for which the research protocols and exposure concentrations are not available for comparison, were summarized by Gates and Renshaw (1946) and are provided in Table 8.3 as LCt_{50} values.

8.3.6.1.2 Sublethal levels

A consistent endpoint for sublethal effects determination is miosis. This information is summarized in Table 8.4.

Van Helden et al. (2003, 2004a,b) exposed male and female marmosets (*Callithrix jacchus*, Harlan, UK) (whole-body) to mean GB vapor concentrations of 0.27−0.91 μg/m^3 and male Dunkin−Hartley guinea pigs to 0.02−0.43 μg/m^3 for 5 h. The lowest cumulative exposure at which the internal dose became measurable (based on fluoride-regenerated GB from blood BuChE) was 0.04 ± 0.01 mg min/m^3 in marmosets and 0.010 ± 0.002 mg min/m^3 in guinea pigs.

Miosis, EEG effects, and visual-evoked response (VER) were examined after 5 h exposures at concentrations ranging from 7.5 to 150 μg GB/m^3. Significant miosis (as measured by the ratio of pupil diameter to iris diameter; $P < .05$) was attained for marmosets and guinea pigs (Table 8.4) (Van Helden et al., 2003, 2004a). Significant ($P < .05$) threshold change in EEG parameters for marmosets occurred at 0.2 mg min/m^3, whereas significant threshold VER changes occurred at 25 mg min/m^3 (Van Helden et al., 2004b).

Mioduszewski et al. (2002a,b) exposed young adult (8- to 10-week-old) male and female Sprague−Dawley (SD) rats to whole-body GB vapor concentrations of 0.01−0.48 mg/m^3 for three exposure periods of 10, 60, and 240 min in a dynamic airflow inhalation chamber. Rat pupil diameters were assessed and blood samples were also collected for RBC−AChE, BuChE, and plasma carboxylesterase (CarbE) activity determinations. Animals were also observed for development of clinical signs during a 7-day postexposure period; EC_{50} values for miosis were reported (Table 8.4; miosis EC_{50} points defined as the statistical concentration required for postexposure pupil diameters of 50% or less of the pre-exposure pupil diameter in 50% of the exposed population) (Mioduszewski et al., 2002a,b). Gender differences (females more susceptible) were observed. Whole-body exposure to GB vapor did not result in significant activity inhibition for any blood enzyme monitored (RBC−AChE, plasma−BuChE, or CarbE) for any GB vapor concentration and duration tested. Mioduszewski et al. (2002a,b) concluded that clinical signs associated with whole-body GB vapor exposure were limited to miosis.

Kassa et al. (2001) exposed male albino Wistar rats for 60 min in an inhalation chamber once, or repeatedly to GB concentrations of 0.8, 1.25, or 2.5 mg/m^3. Animals exposed to the lowest concentration (level 1) were asymptomatic based on clinical and laboratory measurements. Animals exposed to the second concentration (level 2) were asymptomatic based on clinical signs but experienced significant inhibition of RBC−AChE activity

TABLE 8.3 Acute inhalation lethality (LC_{50}; LCt_{50}) for nerve agent vapor in laboratory animals.

Agent	Species	LC_{50} (mg/m^3)	LCt_{50} (mg min/m^3)	Duration (h)	References
GB	Rat (f)	18.1	–	0.16[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	22.6	–	0.16[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	8.51	–	0.50[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	8.84	–	0.50[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	6.39	–	1[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	7.55	–	1[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	4.46	–	1.5[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	4.81	–	1.5[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	3.03	–	4[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	4.09	–	4[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (f)	2.63	–	6[a]	Mioduszewski et al. (2001, 2002a)
GB	Rat (m)	2.89	–	6[a]	Mioduszewski et al. (2001, 2002a)
GB	Mouse (m)	21.5	–	0.33	Bide and Risk (2004)
GB	Mouse (m)	9.0	–	1	Bide and Risk (2004)
GB	Mouse (m)	5.0	–	3	Bide and Risk (2004)
GB	Mouse (m)	3.4	–	6	Bide and Risk (2004)
GB	Mouse (m)	3.1	–	12	Bide and Risk (2004)
GB	Guinea pig (m)	3.99	–	1	Whalley et al. (2007)
GE[b]	Rat	–	260 to <350	0.16	Gates and Renshaw (1946)
GE[b]	Mouse	–	245	0.08	Gates and Renshaw (1946)
GE[b]	Mouse	–	330–1000	0.16	Gates and Renshaw (1946)
GE[b]	Mouse	–	570	0.5	Gates and Renshaw (1946)
GE[b]	Guinea pig	–	>210–1000	0.16	Gates and Renshaw (1946)
GE[b]	Rabbit	–	230–1000	0.16	Gates and Renshaw (1946)
GE[b]	Cat	–	170	0.16	Gates and Renshaw (1946)
GE[b]	Dog	–	230	0.16	Gates and Renshaw (1946)
GE[b]	Monkey	–	210	0.16	Gates and Renshaw (1946)
GF	Rat (f)	25.2	–	0.16[a]	Anthony et al. (2003, 2004)
GF	Rat (m)	36.9	–	0.16[a]	Anthony et al. (2003, 2004)
GF	Rat (f)	5.49	–	1[a]	Anthony et al. (2003, 2004)
GF	Rat (m)	6.60	–	1[a]	Anthony et al. (2003, 2004)
GF	Rat (f)	2.2	–	6[a]	Anthony et al. (2003, 2004)
GF	Rat (m)	2.48	–	6[a]	Anthony et al. (2003, 2004)
GF	Minipig (f)	8.69	–	0.16	Hulet et al. (2006b)
GF	Minipig (m)	7.25	–	0.16	Hulet et al. (2006b)
GF	Minipig (f)	2.12	–	1	Hulet et al. (2006b)
GF	Minipig (m)	1.76	–	1	Hulet et al. (2006b)
GF	Minipig (f)	0.97	–	3	Hulet et al. (2006b)

(*Continued*)

TABLE 8.3 (Continued)

Agent	Species	LC_{50} (mg/m^3)	LCt_{50} (mg min/m^3)	Duration (h)	References
GF	Minipig (m)	1.01	–	3	Hulet et al. (2006b)
VX	Rat (f)	5.44	–	0.16	Benton et al. (2006b, 2007)
VX	Rat (m)	4.85	–	0.16	Benton et al. (2006b, 2007)
VX	Rat (f)	0.74	–	1	Benton et al. (2006b, 2007)
VX	Rat (m)	0.65	–	1	Benton et al. (2006b, 2007)
VX	Rat (f)	0.16	–	4	Benton et al. (2006b, 2007)
VX	Rat (m)	0.16	–	4	Benton et al. (2006b, 2007)

f, Female; *m*, male.
[a]*Lethality assessed over 14 days.*
[b]*LCt_{50} values summarized from numerous obscure sources by Gates and Renshaw (1946).*

(by 30%). The highest test concentration (level 3) was reported to be a nonconvulsive symptomatic exposure. Three months after exposure, control and exposed animals were evaluated for GB-induced effects using biochemical, hematological, neurophysiological, behavioral, and immunotoxicological methods. None of the exposed animals showed any clinical signs of intoxication; their body weight did not differ significantly from control values, and there were no changes in hematological or biochemical parameters, including blood and brain cholinesterase activity. The only significant effect ($P < .05$) observed in rats exposed once to 1.25 mg GB/m^3 (level 2) was an increase in stereotypical behavior. In a continuation of these studies, Kassa et al. (2004) reported that at 3 months after exposure, the level 3 animals showed significant increases in two biochemical markers of stress, plasma corticosterone, and liver tyrosine aminotransferase activities. The latter was also significantly increased in level 2 test animals. In spatial discrimination tests, animals tested at all three GB concentrations showed significant increases in reaction time up to 2 h after exposures. In the level 3 animals, the effects lasted for 3 weeks.

Walday et al. (1991) exposed male Wistar rats to 0.05 or 0.2 mg GD/m^3 for a single 40 h period. No clinical signs of toxicity were seen during the exposures. AChE, BuChE, and CarbE activities were significantly inhibited in airway and lung tissue at both doses. Brain BuChE and CarbE activity exhibited significant effects at either dose; brain AChE activity did not significantly change from baseline at 0.05 mg GD/m^3, but did so at 0.2 mg GD/m^3.

Genovese et al. (2004) evaluated cognitive and general performance effects of GB on adult male SD rats. The test animals received a single whole-body exposure for 60 min once at 1.7−4.0 mg GB/m^3. Cognitive and behavioral performance testing began 48 h after inhalation exposure and was conducted during 55 sessions occurring over approximately 11 weeks after exposures. Single exposures did not significantly affect performance and no delayed performance onset was observed.

Genovese et al. (2008) characterized the miosis EC_{50} for sarin in a nonhuman primate (African green monkey; *Chlorocebus aethiops*) after 10 min exposures (Table 8.4). Evaluation of potential behavioral change by performance on a serial probe recognition test indicated no change from baseline for all subjects. No other clinical sign was observed.

Genovese et al. (2006) also evaluated cognitive and general performance effects of GF on adult male SD rats using the same protocol as that for GB. The test animals were exposed for 60 min to 0, 1.6, 3.7, or 5.2 mg GF/m^3. The highest test concentration resulted in a significant decrease in the response rate in the behavioral task for the first two postexposure sessions; however, the deficit was not persistent and recovery occurred rapidly. None of the exposures to GF caused a significant effect on completion time in the maze (cognitive) task.

In tests conducted by Allon et al. (2005), freely moving male albino SD rats were exposed to whole-body 34.2 ± 0.8 μg GB/L for 10 min, after which electrocardiograms of exposed and control animals were monitored every 2 weeks for 6 months. One and 6 months after exposure, rats were challenged with epinephrine under anesthesia and the threshold for cardiac arrhythmia was determined. Surviving treated rats displayed agitation, aggression, and weight loss compared with nonexposed rats, and approximately 20% experienced sporadic convulsions. GB-challenged rats with severe signs demonstrated QT segment prolongation during the first 2−3 weeks after exposure. Epinephrine-induced arrhythmias appeared at a significantly lower blood pressure in the

TABLE 8.4 Experimental ocular toxicity (miosis) values for G-agents and VX vapor exposures.

Species	Toxicity value						References
	GB (mg/m^3)	GB (mg min/m^3)	GD (mg min/m^3)	GF (mg/m^3)	GF (mg min/m^3)	VX (mg/m^3)	
Human (10 min–5 h, ECt_{90})	–	13.85	–	–	–	–	Callaway and Dirnhuber (1971)
Human (20 min, ECt_{50})	–	4	–	–	–	–	Johns (1952)
Human (10 min–5 h, ECt_{50})	–	2.33	–	–	–	–	Callaway and Dirnhuber (1971)
Human (20 min, no effect)	–	1.2	–	–	–	–	McKee and Woolcott (1949)
Marmoset (5 h, threshold)	–	2.5	–	–		–	Van Helden et al. (2004a)
Rabbit (10 min–5 h, ECt_{50})	–	1.32	0.59	–	0.75[a]	–	Callaway and Dirnhuber (1971)
Rabbit (10 min–5 h, ECt_{90})	–	2.71	2.19	–	1.79[a]	–	Callaway and Dirnhuber (1971)
Guinea pig (5 h, threshold)	–	1.8	–	–	–	–	Van Helden et al. (2004a)
Rat (m) (10 min, EC_{50})	0.087	–	–	0.184	–	0.01	Benton et al. (2005, 2006a)
Rat (m) (60 min, EC_{50})	0.030	–	–	0.042	–	0.004	Benton et al. (2005, 2006a)
Rat (m) (240 min, EC_{50})	0.024	–	–	0.029	–	0.002	Benton et al. (2005, 2006a)
Rat (f) (10 min, EC_{50})	0.068	–	–	0.080	–	0.007	Benton et al. (2005, 2006a)
Rat (f) (60 min, EC_{50})	0.020	–	–	0.024	–	0.002	Benton et al. (2005, 2006a)
Rat (f) (240 min, EC_{50})	0.012	–	–	0.017	–	0.001	Benton et al. (2005, 2006a)
Göttingen minipig (m) (10 min, EC_{50})	0.244	–	–	0.161	–	–	Hulet et al. (2006a)
Göttingen minipig (m) (60 min, EC_{50})	0.043	–	–	0.047	–	–	Hulet et al. (2006a)
Göttingen minipig (m) (180 min, EC_{50})	0.032	–	–	0.022	–	–	Hulet et al. (2006a)
Göttingen minipig (f) (10 min, EC_{50})	0.214	–	–	0.190	–	–	Hulet et al. (2006a)
Göttingen minipig (f) (60 min, EC_{50})	0.044	–	–	0.058	–	–	Hulet et al. (2006a)
Göttingen minipig (f) (180 min, EC_{50})	0.022	–	–	0.037	–	–	Hulet et al. (2006a)
African green monkey (f) (10 min, EC_{50})	0.469	–	–	–	–	–	Genovese et al. (2008)

f, female; *m*, male.
[a]*Data for T2715 (2-methylcyclohexyl methylphosphonofluoridate) analog for agent GF.*

treated group during the first month after exposure and lasted for up to 6 months.

Callaway and Dirnhuber (1971) evaluated the miotogenic potency of GB vapor in rabbits exposed to GB under goggles (43 miosis responses in 10 albino rabbits). Exposure time periods ranged from 10 min to 5 h (Table 8.4).

Bartosova-Sevelova and Bajgar (2005) exposed rats to agent GB vapors for 4 h at four different concentrations (0.30, 0.43, 0.58, and 0.82 mg/m^3) in a whole-body exposure chamber. Convulsions and hypersalivation were observed in one animal exposed to 0.82 mg/m^3. There was a significant decrease in blood AChE activity in all but the low-dose test groups and the controls. AChE activity in the brain was significantly decreased only in animals exposed to 0.58 mg/m^3, and only in the pontomedullar area. No significant alterations in AChE activity were seen in the frontal cortex or in the basal ganglia. AChE activity in the pontomedulla was lowest at the greatest dose (0.82 mg/m^3), but the data were too variable for statistical significance.

Sekowski et al. (2004) evaluated gene and protein level changes in the brains of male and female SD rats exposed to low-level doses (0.004−0.033 mg/m^3) of aerosolized agent GB and GF via whole-body inhalation for 4 h. Preliminary results indicate that exposure to nerve agents results in differential expression of a number of neuronal genes, including a group that affects cellular processes critical to neurological injury and regeneration, and gender-associated differences in the level and type of gene expression response were significant.

Whalley et al. (2004) exposed adult male and female SD rats to a series of whole-body agent GF vapor concentrations for 10, 60, or 240 min. Miosis (defined as a 50% reduction in pupil area compared with baseline) measured approximately 30 min after exposure indicates that females were significantly more sensitive than males ($P < .05$) (Table 8.4).

In studies conducted by Hulet et al. (2006a,c, 2007), male and female Göttingen minipigs were exposed to whole-body agent GB or GF for 10, 60, or 180 min (Table 8.4). Male minipigs were significantly ($P = .022$) more sensitive to the effects of GF exposure than females.

Conn et al. (2002) exposed male F344 rats to 0, 0.2, or 0.4 mg GB/m^3 for 1 h/day for 1 or more days. Animals were maintained at either 25°C or 32°C to evaluate the effects of heat stress. Body temperature and activity were monitored by telemetry continuously during exposure and for 1 month after the exposures. Although RBC−ChE activity was reduced in the exposed animals (quantitative data not provided), the test protocol did not significantly alter temperature regulation or locomotive activity of the rats.

8.3.6.2 Agent VX

8.3.6.2.1 Lethal levels

Benton et al. (2006b, 2007) experimentally determined the LCt_{50} and LC_{50} in male and female adult SD rats exposed to whole-body VX vapor for 10, 60, and 240 min in a dynamic exposure chamber (Table 8.3); the study protocol was similar to that for agent GB in the studies conducted by Mioduszewski et al. (2001, 2002a). Experiments testing the role of decontamination less than 24 h after exposure provided clear evidence for percutaneous toxicity induced by whole-body vapor exposure to the persistent nerve agent VX. For severe and lethal VX vapor exposure effects, females were not more susceptible than males for the exposure durations examined.

Bide and Risk (2000) exposed outbred male CD1 (SD) BR rats, outbred male CD1 (ICR) BR mice, and outbred male (HA) BR guinea pigs to NaCl aerosols containing entrained VX in a nose-only inhalation system for an exposure time of 12 min. Observed LCt_{50} values are summarized in Table 8.3.

Bide and Risk (2000, 2004) also cite several previous studies in which LCt_{50} values for mice, guinea pigs, rabbits, hamsters, and dogs were reported (Table 8.3).

For exposure to VX vapors, Koon et al. (1960) reported 10 min LCt_{50} values for mice with exposure to either whole-body or head only, as well as for goats. Carroll et al. (1957) also reported female mouse LCt_{50} values for nose-only and whole-body protocols. However, Carroll et al. (1957) reported that the concentration of VX in the exposure chamber was not measured directly but was estimated from the mortality level, which was correlated with the LD_{50} for i.v. injection.

8.3.6.2.2 Sublethal level

Benton et al. (2005, 2006a, 2007) have characterized miosis as well as severe effects (severe tremors and/or prostration, convulsions, and/or gasping) in male and female SD rats exposed to whole-body VX vapor (0.00037−0.016 mg VX/m^3) under study protocols similar to those for agent GB in the studies conducted by Mioduszewski et al. (2001, 2002a). Miosis EC_{50} endpoints were derived for VX vapor exposure durations of 10, 60, and 240 min (Table 8.4). At the highest VX concentrations tested for each exposure duration, significant (>99.9% confidence) differences between control and experimental whole-blood AChE activity were observed; no other signs (e.g., tremors, salivation) were observed and delayed pupil effects were minimal. For the miosis endpoint, female rats are considered more susceptible than males to VX vapor exposure.

For severe effects (tremors, prostration, etc.), the ECt_{50} values (mg min/m^3) reported by Benton et al. (2007) were

as follows: 10 min, 40.9 (female) and 35.2 (male); 60 min, 30.0 (female) and 31.2 (male); and 240 min, 31.5 (female) and 29.9 (male). EC concentrations were not reported.

After single 60 min VX vapor exposures in the range of 0.016–0.45 mg VX/m^3, Genovese et al. (2007) examined blood AChE activity, dose estimation by regeneration assay, transient miosis, and behavior parameters in adult male SD rats. Behavioral evaluation included a radial maze task and a variable-interval schedule-of-reinforcement task. At all concentrations tested, transient miosis and AChE activity inhibition were observed and some subjects exhibited transient ataxia and slight tremor. After 3-month postexposure evaluations of behavior, the authors concluded that performance deficits were minor and transient at these concentrations. Further, no delayed effects were observed.

8.4 Risk assessment

Application of standard risk assessment methods by numerous authorities and agencies to the toxicological data summarized has generated exposure guidelines that provide objective and health-based foundations for responsible and efficient responses after nerve agent release, as well as a basis for site recovery and decontamination decision criteria. The health-based nerve agent exposure guidance summarized here has been derived in an open and transparent manner and judged scientifically valid and protective (Opresko et al., 1998, 2001; NRC, 1999, 2001, 2003; Krewski et al., 2004; Watson et al., 2006a,b; see also www.epa.gov/oppt/aegl/). Although initially developed to facilitate disposal of the US stockpile of CW munitions and to support remediation or closure at sites where CWs were historically processed, nerve agent exposure guidance became a subject of interest for homeland defense applications after the events of September 2001.

For reasons described previously, the air exposure pathway has been a primary focus of risk assessment activity for nerve agents (NRC, 2003; Cannard, 2006; ATSDR, 2007; Dabisch et al., 2008a). In situations where long-term agent release is a concern, and where agent residuals may be found, potential exposure to relatively low levels of ingested agents is a priority.

In all cases, it is important to appropriately safeguard public health without defaulting to overly conservative actions (e.g., to "nondetect") that would divert limited resources without significant benefit. The following sections summarize toxicological support and developmental rationale for the two primary criteria of interest to community decision-makers managing response to an intentional or accidental release of nerve agent(s) to the environment.

8.4.1 Acute exposure guideline levels

Credible short-term nerve agent exposure limits, designed to aid state and local government agencies in developing emergency response plans in the event of accidental or deliberate atmospheric release, have been derived. These short-term values have also proved useful in deployed force health protection, and in establishing health-based CWA performance goals for detection system development (USACHPPM, 2004, 2008).

Acute exposure guideline levels (AEGLs; expressed in units of mg/m^3 or ppm) are vapor exposure guideline values for numerous hazardous compounds (primarily toxic industrial compounds) that have been published by the National Academy Press (e.g., NRC, 2003, 2007). For each hazardous compound, guideline levels are developed for vapor exposure durations of 10 and 30 min, 1, 4, and 8 h, as well as for three gradations of toxic effect severity. AEGL-1 concentrations are the mildest effect category, whereas AEGL-3 concentrations represent the most severe effect category (NRC, 2001). The point above the AEGL-3 concentration at which level 3 effects would initiate for any given human exposure duration is not identified in the AEGL assessment protocol.

Typically, the AEGL concentration established for any given effect level is often less than the known experimental concentration at which such toxicological effects occur. This protective nature of the AEGL process and values was demonstrated for each of the nerve agents, where observed human thresholds for reversible effects occur at air concentrations greater than AEGL-1 levels (Watson et al., 2006a,b).

Selection protocols for critical effects and studies, AEGL derivation, time scaling, use and selection of uncertainty and modifying factors, and a description of the lengthy and deliberative review process used are all described in NRC (2001) as well as in recent articles by Krewski et al. (2004) and Bruckner et al. (2004). Development of AEGL values includes consideration of uncertainty factors as well as the need for any modifying factors. Because exposure–response data are usually not available for each AEGL-specific exposure duration (NRC, 2001), temporal extrapolation is used in the development of values for some AEGL-specific time periods. The concentration–exposure time relationship for many systemically acting vapors and gases may be described by $Cn \times t = k$, where the exponent n ranges from 0.8 to 3.5 (Ten Berge et al., 1986; NRC, 2001). Some investigators refer to the Ten Berge extrapolation as the "toxic load model" and n as the "toxic load exponent" (Sommerville et al., 2006; Dabisch et al., 2008a). The excellent data collected by investigators at Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), characterizing nerve agent vapor exposure miosis and lethality

endpoints for multiple agents and species, has allowed agent-specific determination of *n* (summarized in Dabisch et al., 2008a). In the case of swine exposed to G-agents and rats exposed to VX, the experimentally determined *n* values for these endpoints are 1.6 or less, which are less than the *n* of 2 assumed during AEGL development for these same compounds in 2001–2003 (NRC, 2003), and based on then-available data for SD rats exposed to agent GB (Mioduszewski et al., 2002a,b). It thus appears that G-agent and VX dose response for the miosis and lethality endpoints are less steep than previously indicated and that the published nerve agent AEGL values (Table 8.5) are more protective than originally considered (NRC, 2003; Watson et al., 2006a,b).

For comparison, it is useful to consider other common guideline sources applicable to short-term nerve agent release events. The US Department of Energy, in their development of 1 h Protective Action Concentrations/Temporary Emergency Exposure Levels (PAC/TEELS), has chosen to replicate the published nerve agent-specific 1 h AEGL-1, AEGL-2, and AEGL-3 values as tier 1, tier 2, and tier 3 PAC/TEEL values, respectively (http://hss.energy.gov/HealthSafety/WSHP/chem_safety/teel.html).

8.4.1.1 Application of AEGL values

The AEGL process does not include specific implementing or application guidance, and specific approaches for using the values are left to the discretion of risk managers and appropriate authorities (NRC, 2001). Nevertheless, AEGL application is already broad and continues to expand.

The utility of AEGL values for CW agent emergency preparedness planning was recognized by the Chemical Stockpile Emergency Preparedness Program (CSEPP), when FEMA and US Army representatives adopted final nerve agent AEGL concentrations to replace previous agent toxicity criteria for emergency response decision making (CSEPP, 2003). As of February 2003, standing CSEPP policy guidance for each of the communities hosting agent demilitarization facilities in the US, recommends application of AEGL-2 concentrations as the protective action level for evacuation or shelter-in-place, and application of AEGL-1 concentrations as notification levels (CSEPP, 2003). Since publication of final AEGL levels by NRC (2003) and enactment of the CSEPP Policy Paper (CSEPP, 2003), multiple stockpile states and counties have incorporated the Policy Paper

TABLE 8.5 Summary of AEGL values for G-series nerve agents and VX (mg/m^3).

Agent	Classification	10 min	30 min	1 h	4 h	8 h
GA	AEGL-1	0.0069	0.0040	0.0028	0.0014	0.0010
	AEGL-2	0.087	0.050	0.035	0.017	0.013
	AEGL-3	0.76	0.38	0.26	0.14	0.10
GB	AEGL-1	0.0069	0.0040	0.0028	0.0014	0.0010
	AEGL-2	0.087	0.050	0.035	0.017	0.013
	AEGL-3	0.38	0.19	0.13	0.070	0.051
GD	AEGL-1	0.0035	0.0020	0.0014	0.00070	0.00050
	AEGL-2	0.044	0.025	0.018	0.0085	0.0065
	AEGL-3	0.38	0.19	0.13	0.070	0.051
GF	AEGL-1	0.0035	0.0020	0.0014	0.00070	0.00050
	AEGL-2	0.044	0.025	0.018	0.0085	0.0065
	AEGL-3	0.38	0.19	0.13	0.070	0.051
VX	AEGL-1	0.00057	0.00033	0.00017	0.00010	0.000071
	AEGL-2	0.0072	0.0042	0.0029	0.0015	0.0010
	AEGL-3	0.029	0.015	0.010	0.0052	0.0038

Source: Adapted from National Research Council (NRC), 2003. Committee on Toxicology, Board on Environmental Studies and Toxicology, Commission on Life Sciences. The National Academies Press, Washington, DC with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington, DC.

TABLE 8.6 Estimated reference doses (RfDe), RfD uncertainty factors, and health-based environmental screening levels for nerve agents.

Nerve agent	RfDe (mg/kg/day)	Composite uncertainty	Residential soil preliminary remediation goal (mg/kg)	Industrial soil preliminary remediation goal (mg/kg)
VX	6E-7	100	0.042 (est.)	1.1 (est.)
GA	4E-5	3000	2.8 (est.)	68.0 (est.)
GB	2E-5	3000	1.3 (est.)	32.0 (est.)
GD	4E-6	3000	0.22 (est.)	5.2 (est.)

Source: From Opresko, D.M., Young, R.A., Faust, R.A., 1998. Chemical warfare agents: estimating oral reference doses. Rev. Environ. Contam. Toxicol. 156:1–183; Opresko, D.M., Young, R.A., Watson, A.P., 2001. Chemical warfare agents: current status of oral reference doses. Rev. Environ. Contam. Toxicol. 172, 65–85; US Army Center for Health Promotion and Preventive Medicine (USACHPPM), 1999. Derivation of Health-Based Environmental Screening Levels for Chemical Warfare Agents: A Technical Evaluation. US Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD; Office of the Assistant Secretary of the Army (OASA), 1999. Derivation of Health-Based Environmental Screening Levels (HBESL) for Chemical Warfare Agents. Memorandum signed by Raymond J. Fatz, Deputy Assistant Secretary of the Army, May 28, 1999. Department of the Army, Office of the Assistant Secretary (Environment, Safety, and Occupational Health), Army Pentagon, Washington, DC; Watson, A.P., Dolislager, F.G., 2007. Reevaluation of 1999 Health-Based Environmental Screening Levels (HBESLs) for Chemical Warfare Agents ORNL/TM-2007/080. Oak Ridge National Laboratory, Oak Ridge, TN.

recommendations into their individual community emergency response plans, and used them in making regulatory decisions permitting agent munition disposal operations (CSEPP, 2006a,b).

In February 2008, the US National Response Team (NRT) posted Quick Reference Guides for the G-series nerve agents and VX for public access on its website (www.nrt.org) (NRT, 2008). These Quick Reference Guides are useful summaries of agent characteristics and advisories and are provided as national guidance. While acknowledging that site-specific clean-up decision criteria will be the result of multiagency agreements and site-specific factors, the NRT considers that attainment of agent-specific air concentrations less than 8 h of AEGL-1 is an acceptable criterion for verification of site decontamination.

In general, agent concentrations less than AEGL-2 are considered to be in a range that poses relatively negligible health consequences for acute exposures. Other AEGL applications performed or recommended include use as hazard assessment plume modeling criteria for the US Nonstockpile Chemical Material Program and Homeland Defense scenarios, as testing criteria for personal protective equipment intended for use by first responders in a single weapon-of-mass-destruction scenario, as detection performance goals for advanced equipment acquisition and development, and as a tool for assessing potential exposures during military missions such as peacekeeping (USACHPPM, 2008).

8.4.2 Estimated oral reference doses

Development of nerve agent-specific reference dose estimates is critical to remediation and restoration at existing and closing military sites, which is a priority activity for the Department of Defense (DOD) (Opresko et al., 1998, 2001). A reference dose (RfD; mg/kg/day) was originally designed for estimating noncancer health risks at CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act of 1980) Superfund sites (USEPA, 1989). As such, it is an essential component of the site risk assessment used to assess potential long-term exposures to contaminated media such as soil, where RfDs address the pathway of incidental ingestion of soil particles (USEPA, 1989; Dourson et al., 1994; Cicmanec et al., 1996; Abernathy et al., 2004).

Methods used to derive oral RfDs for nerve agents follow standard USEPA protocols (USEPA, 1989; Dourson et al., 1994), use appropriate toxicological data and uncertainty factors, and have undergone review for consistency by the National Research Council (Opresko et al., 1998, 2001; NRC, 1999; Bakshi et al., 2000). Because the EPA has not officially verified the derived values for nerve agents, they are identified as estimated RfDs (RfDe) (Table 8.6).

These criteria are selected by the Office of the Army Surgeon General as the most appropriate oral toxicity reference values for use in environmental risk assessments and represent the Army's position (Opresko et al., 2001). The RfDe values have been put in to USEPA risk models along with accepted chronic vapor exposure limits (as cited in Watson and Dolislager, 2007) to generate agent-specific Health-Based Environmental Screening Levels (HBESLs). These HBESLs are endorsed by military policy as criteria to assess potentially contaminated soils (Table 8.6) (USACHPPM, 1999; OASA, 1999; Watson and Dolislager, 2007).

8.4.3 Management of exposure to nerve agents

The continued use of nerve agents by terrorists and militant states necessitates the need for further effort in the management of exposure to these agents. This entails decontamination of exposure sites, prophylactic measures, and medical intervention to treat both immediate and longer-term effects of nerve agents. Although briefly considered here, these topics are addressed more fully in later chapters of this volume.

8.4.4 Critical role of decontamination

Before discussion of antidotes and treatment regimens, it is noted that affected individuals should be removed from the site of agent exposure as quickly as possible, and undergo rapid decontamination to remove potential for continued personal exposure and to prevent secondary exposure to responders, healthcare workers, medical transport vehicles, and treatment facilities (Sidell et al., 1997; Cannard, 2006; ATSDR, 2007; Okumura et al., 2007; Pulley et al., 2008; Chilcott et al., 2019). Decontamination efforts generally focus on removing agent from the skin and limiting further contact from agent-contaminated clothing and other contaminated materials. It is imperative that decontamination of the skin be rapid, efficacious and, especially for those involved in armed conflicts, easily performed. Over the years, decontaminants have included the M291 Skin Decontamination Kit (SDK), Reactive Skin Decontamination Lotion (RSDL), soapy water, and dilute sodium hypochlorite among others.

8.4.5 Signs and symptoms guiding medical management

Depending on the concentration and duration of exposure, cases of nerve agent intoxication can exhibit a dose-dependent "constellation" of clinical signs and symptoms representing a variety of parasympathetic effects, functional change at NMJs, and altered CNS function (Cannard, 2006). As a consequence, critical care personnel and others responsible for developing and administering treatment protocols should take into account the totality of the case presentation. A good example is drawn from observations made by medical personnel treating subway passengers presenting at Tokyo area hospitals and clinics on day 1 of the sarin release incident. In decreasing order of frequency, the following clinical signs and symptoms were noted: miosis (observed in most patients), headache, dyspnea, nausea, vomiting, muscular weakness, cough, rhinorrhea, chest oppression, muscular fasciculations, and psychological disturbances such as anxiety (Lillibridge, 1995). Similar distributions were observed among subway passengers treated at St. Luke's International Hospital (Okumura et al., 1996, 2007) as well as residents affected in the Matsumoto incident of 1994 (Yanagisawa et al., 2006), and are consistent with classic descriptions of nerve agent intoxication (Sidell et al., 1997; Leikin et al., 2002; Cannard, 2006). Miosis was found to be a more responsive exposure index than RBC−ChE activity inhibition (Nozaki et al., 1997) or serum cholinesterase activity (Yanagisawa et al., 2006) in cases of sarin vapor exposure during the Tokyo and Matsumoto incidents, respectively (Okumura et al., 2015).

Yanagisawa et al. (2006) classified individuals exhibiting a pupil diameter larger than 3.0 mm as without miosis and not affected by nerve agent vapor exposure given that simultaneous serum ChE activity measurements taken during treatment of the Matsumoto cases displayed no inhibition (e.g., largely $\geq 100\%$ of normal serum ChE activity with two cases exhibiting activity $<90\%$ of normal). This, and additional miosis data from Matsumoto, can be used in determining appropriate treatment for individuals without known or confirmed nerve agent vapor exposure (Cannard, 2006; Yanagisawa et al., 2006); for these individuals, examination, observation without treatment, and discharge are appropriate actions and were successfully implemented during medical responses to the Tokyo subway incident (Lillibridge, 1995; Okumura et al., 2015).

8.4.6 Nerve agent antidotes

Advances in medical countermeasures for OP nerve agents are a continuing area of research. The choice of a specific treatment regimen depends on the agent as well as the extent and route(s) of exposure. Very mild exposure to nerve agent vapor may necessitate only decontamination and observation; severe exposure to vapor or liquid requires immediate decontamination, antidote administration, artificial respiration, monitoring, and supportive therapy over hours to multiple days (Sidell et al., 1997; ATSDR, 2007; Vale et al., 2007; Pulley et al., 2008). Convenient triage classifications have been developed by ATSDR (2007) in collaboration with the US Army Medical Research Institute of Chemical Defense.

In cases of massive inhalation exposure, immediate care is vital to prevent death from respiratory failure and because the agent−AChE complex becomes resistant to reactivation by oxime-type antidotes. "Aging" is compound-specific; aging half-times range from minutes (agent GD) to days (agent VX) (Sidell and Groff, 1974; Sidell et al., 1997). Standard antidotes clinically available in the United States are atropine (anticholinergic) and pralidoxime (Protopam or 2-PAM-Cl). In addition, CNS active drugs such as diazepam (Valium) are strongly recommended if convulsions occur; anticonvulsant

treatment is critical for protection against lethality and brain pathology (Shih et al., 2003). If administered soon after exposure, benzodiazepine anticonvulsants reduce seizures and improve recovery but may also increase the risk of seizure recurrence (Figueiredo et al., 2018).

Individuals exposed to vapor and exhibiting miosis only or miosis and rhinorrhea only do not usually require antidote treatment and will resolve without medical intervention (Cannard, 2006; ATSDR, 2007), but they should be observed. If rhinorrhea is problematic in these vapor-only cases, then ATSDR (2007) advises intramuscular atropine (0.05 mg/kg pediatric; 2.0 mg adult) to relieve signs, followed by patient discharge. If eye pain/headache or nausea is problematic in vapor-only cases, then ATSDR (2007) further advises administration of topical atropine or homatotropine to the eye for relief, eye protection from bright light, and discharge.

If liquid/droplet exposure is known or suspected in an individual exhibiting miosis only or miosis and rhinorrhea only, it is recommended that the individual receive no antidote treatment but be closely observed for at least 18 h, because toxic effects of liquid percutaneous exposure may not manifest for several hours (Sidell et al., 1997; Cannard, 2006). Toxic effects from vapor-only exposure usually occur quickly (within minutes) (Sidell et al., 1997). Current medical management guidelines and recommended medication protocols are summarized in ATSDR (2007), Vale et al. (2007), and Pulley et al. (2008). The ATSDR antidote treatment protocol for civilian emergency management is summarized in Table 8.7. The reader may also wish to consult the National Institute for Occupational Safety and Health (NIOSH) (CDC, 2019) for antidote information. Additional information regarding medical management protocols is discussed in Chapters 67, 68, and 71 of this volume.

8.4.7 Ongoing antidote development

The development of medical countermeasures to OP nerve agents continues to receive significant attention. Given that termination of seizure activity protects against the development of neuropathological lesions (especially neuronal necrosis) in brain tissues of experimental animals (Martin and Doebler, 1985; Shih et al., 2003; Marrs et al., 2007b), focus on anticonvulsant therapy is critical. Reduced potential for permanent brain damage in human cases by preventing or limiting the duration of status epilepticus is a primary goal. In challenge tests against multiple LD_{50} doses of agents GA, GB, GD, GF, VX, and VR in guinea pigs, the anticonvulsants midazolam and trihexyphenidyl were more effective than diazepam for seizure control, and midazolam was the most rapidly effective (Shih et al., 2003). The diazepam pro-drug avizafone is also effective (Lallement et al., 2000, 2004) and is available via autoinjector administration (as is diazepam). For more rapid seizure control during acute treatment phases, Marrs et al. (2007b) recommend midazolam administered intramuscularly because of its more rapid intramuscular absorption.

Although pralidoxime is an effective and well-tolerated reactivator, its potency is limited. The search has thus continued for oximes that would combine high reactivator effectiveness against nerve agents but have low toxicity and good chemical stability. Initially, several promising drugs (the oxime HI-6; trimedoxime, or TMB-4; and obidoxime, or LüH-6) emerged but are not equally effective against all agents and forms of exposure (Dawson, 1994; Marrs et al., 2006; Antonijevic and Stojiljkovic, 2007; Eyer et al., 2007; Wetherell et al., 2007; Kuca et al., 2009; Kassa et al., 2010). Marrs et al. (2006) point out that, in the absence of pyridostigmine pretreatment, there are at present "no clinically important differences" between the standard oxime pralidoxime and the alternative oximes HI-6 and obidoxime in the treatment of nerve agent intoxication. These pyridinium oximes, however, are unable to effectively penetrate the blood−brain barrier and, therefore, are ineffective at reactivating OP-inhibited AChE in the brain (Shih et al., 2012). Ongoing research with novel brain-penetrating oximes (substituted phenoxyalkyl pyridinium oximes) and a sarin surrogate (nitrophenyl isopropyl methylphosphonate) or paraoxon (the active metabolites of the OP pesticide parathion) have shown these oximes to be effective reactivators of OP-inhibited brain AChE (Chambers et al., 2012; Chambers and Meek, 2016; Chambers and Meek, 2019; Dail et al., 2019). These are further discussed elsewhere in this volume.

As a combinatorial drug with atropine, galantamine has been effective and safe in counteracting lethal GD and GB doses in the guinea pig (Albuquerque et al., 2006; Pereira et al., 2008); galantamine also protects against neurodegeneration at doses of LD_{50} or more in the guinea pig, and it shows promise as a pretreatment before GD or GB exposures. Lethality was prevented by treatment with nasal atropine (atropine methyl nitrate), and postexposure treatment with atropine methyl bromide instillation, in combination with pulmonary therapeutic surfactants or liquevents in guinea pigs exposed to approximate LC_{50} concentrations of VX aerosol (Nambiar et al., 2007).

The use of bioscavengers has been considered for both the prevention and treatment of nerve agent-induced toxic effects and research on these continues (Nachon et al., 2013; Masson and Nachon, 2017; Goldsmith and Ashani, 2018). Pretreatment with an anticholinesterase carbamate, such as pyridostigmine bromide (PB), was shown to reversibly sequester (and thereby protect) a fraction of AChE from bonding with circulating nerve agents. The

TABLE 8.7 Recommended antidote protocol for emergency nerve agent exposure therapy.

Patient age	Antidotes		Other treatment
	Mild/moderate signs and symptoms[a]	Severe signs and symptoms[b]	
Infant (0–2 years)	Atropine: 0.05 mg/kg i.m. or 0.02 mg/kg i.v.	Atropine: 0.1 mg/kg i.m. or 0.02 mg/kg i.v.	Assisted ventilation as needed. Repeat atropine (2 mg i.m. or 1 mg i.m. for infants) at 5–10 min intervals until secretions have diminished and breathing is comfortable or airway resistance has returned to near normal. Phentolamine for 2-PAM-induced hypertension (5 mg i.v. for adults; 1 mg i.v. for children). Diazepam for convulsions (0.2 to 0.5 mg i.v. for infants up to 5 years; 1 mg i.v. for children > 5 years; 5 mg i.v. for adults).
	2-PAM-Cl: 15 mg/kg i.v. slowly	2-PAM-Cl: 15 mg/kg i.v. slowly	
Child (2–10 years)	Atropine: 1.0 mg i.m.	Atropine: 2.0 mg i.m.	
	2-PAM-Cl: 15 mg/kg i.v. slowly	2-PAM-Cl: 15 mg/kg i.v. slowly	
Adolescent (> 10 years)	Atropine: 2.0 mg i.m.	Atropine: 4.0 mg i.m.	
	2-PAM-Cl: 15 mg/kg i.v. slowly	2-PAM-Cl: 15 mg/kg i.v. slowly	
Adult	Atropine: 2.0–4.0 mg i.m.	Atropine: 6.0 mg i.m.	
	2-PAM-Cl: 15 mg/kg (1 g) i.v. slowly	2-PAM-Cl: 15 mg/kg i.v. slowly	
Elderly, frail	Atropine: 1.0 mg i.m.	Atropine: 2.0 mg i.m.	
	2-PAM-Cl: 5–10 mg/kg i.v. slowly	2-PAM-Cl: 5–10 mg/kg i.v. slowly	

[a] *Mild/moderate signs and symptoms include localized sweating, muscle fasciculations, nausea, vomiting, weakness, dyspnea.*
[b] *Severe signs and symptoms include unconsciousness, convulsions, apnea, flaccid paralysis.*
Source: Contents reproduced from Agency for Toxic Substances and Disease Registry (ATSDR), 2007. Medical Management Guidelines for Nerve Agents: Tabun (GA), Sarin (GB) Soman (GD) and VX. US Department of Health and Human Services, Washington, DC (public domain).

carbamate moiety spontaneously hydrolyzes from the AChE molecule within hours and allows AChE to again become available for normal physiological function. Such a pretreatment concept and drug enhances the effectiveness of atropine and oximes in treating lethal doses of GA, GB, and VX (Gall, 1981; Inns and Leadbeater, 1983). US combat units already supplied with atropine and pralidoxime have been equipped with 30 mg PB tablets for oral administration every 8 h; the current maximum pretreatment period is 21 days (Sidell et al., 1997; Scott et al., 2007). A related carbamate, physostigmine, was shown to protect animals against not only nerve agent lethality but also incapacitation (Leadbeater et al., 1985). When co-administered with hyoscine, physostigmine effectively reduced incapacitation and prevented death in guinea pigs exposed to agent GD (Wetherell, 1994), and prevented lethality and reduced or prevented incapacitation in guinea pigs exposed to GA, GB, GD, GF, and VX (Wetherell et al., 2002). Transdermal patch administration of physostigmine and hyoscine, or physostigmine alone, in the guinea pig has protected against GD intoxication (Meshulam et al., 1995).

Other developmental pretreatment options included NMDA receptor antagonist memantine in combination with atropine sulfate (Gupta and Dettbarn, 1992; McLean et al., 1992), pre-exposure loading with an excess of

circulating ChE or BuChE (Bajgar et al., 2007; Lenz et al., 2008; Podoly et al., 2008; Saxena et al., 2008; Van der Schans et al., 2008; Masson and Gupta, 2015), or CarbEs (Maxwell et al., 1987) to bind the nerve agent before the agent can reach tissue AChE sites.

Research on the use of bioscavengers such as organophosphate hydrolases is promising for the development of prophylactic measures as well as postexposure treatment of OP nerve agent-induced intoxication (Trovaselet-Leroy, et al., 2011; Masson and Nachon, 2017; Goldsmith and Ashani, 2018). Bioscavengers may interact with OP nerve agents (and OP pesticides) stoichiometrically or catalytically before the OP agents can reach their acetylcholinesterase target. The stoichiometric bioscavengers are proteins that sequester the OP agents while the catalytic bioscavengers bind to and subsequently hydrolyze the OP nerve agent. Multiple examples of these bioscavengers are being investigated individually or as a combination therapeutic measure against OP nerve agent effects. Bioscavengers may offer the potential for pretreatment where the threat of nerve agent exposure exists.

8.5 Concluding remarks and future directions

Stockpiles of OP nerve agents have already been destroyed or are scheduled for destruction in the near future. However, attacks with OP nerve agents by terrorist organizations or by rogue militant states necessitate continued work on protection prior to suspected exposure as well as postexposure medical countermeasures. Medical management of casualties in the event of an attack with OP nerve agents is relevant not only for immediate emergency response but also for longer-term follow up. The latter is being assisted by continued research focusing on identifying and understanding health effects beyond the initial cholinergic crisis syndrome.

Technical advances in precision detection of OP nerve agents further direct attention toward responsible consideration of the consequences for transient presence of residual, low-level agent concentrations in a number of scenarios. Having a transparent, robust, and data-supported framework within which to evaluate likely consequences of potential low-level nerve agent exposure will greatly aid in emergency planning and emergency response operations, and for reducing the magnitude of disruption to affected communities and facilities.

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Chapter 9

Russian VX

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9.1 Introduction and background

One of the most abundant and most toxic agents in the chemical arsenals of the United States and former Soviet Union were VX and VR (Russian VX), respectively, whose development in the middle of the 20th century indicated the peak of warfare chemistry. V-gases are low-volatile liquids with high boiling points, so they are much more persistent than the higher-volatility organophosphorus nerve agents, such as sarin and soman. V-series compounds are more toxic that other organophosphates (OPs). For example, in comparison with sarin (GB), VX is estimated to be approximately twice as toxic by inhalation, 10 times as toxic by oral administration, and approximately 170 times as toxic by skin exposure (Munro et al., 1994). V-series nerve agents are quite effective when exposed through skin contact (especially as tiny drops) and commonly cause death. Poisoning occurs regardless of exposure method, specifically: inhalation; ingestion of vaporous and liquid agents through intact or injured skin or eye mucosa; and contact with contaminated surfaces.

The name *VX* relates to a group of *O,S*-diesters of methylphosphonic acid $ROPO(CH_3)S(CH_2)_2N(R1)_2$. *O*-isobutyl *S*-2-(diethylamino)ethyl methylphosphonothioate (R = *i*Bu, R1 = Et), produced since 1972 exclusively in the former Soviet Union, was generally referred to as *Russian VX* or *VR* (CAS #159939-87-4). The synonyms for this substance are as follows:

- VA;
- Phosphonothioic acid methyl-, *S*-[2-(diethylamino)ethyl] *O*-(2-methylpropyl) ester;
- *O*-isobutyl *S*-2-(diethylamino)ethyl methylthiophosphonate;
- *O*-isobutyl *S*-(*N,N*-diethylaminoethyl) methylphosphonothioate;
- Russian V-gas.

The brutto formula of VR is $C_{11}H_{26}NO_2PS$ (MW 267.368). The structural formula of VR is presented in Fig. 9.1.

VR is a colorless transparent liquid resembling glycerol in mobility, with a boiling point of 300°C, melting point of 35°C, ρ^{20} of 1.0083 g/cm^3, and volatility of C_{max}^{20} 0.0105 mg/dm^3. The agent is poorly soluble in water (less than 5% at 20°C) and highly soluble in organic solvents. Technical products can be colored from yellow to dark brown and smell like fried sunflower seeds.

Research into the environmental behavior of highly toxic chemicals is an important branch of analytical toxicology. We take the word *behavior* to mean the persistence, mechanisms of possible transformation, composition of such transformation products, and toxicity of these substances. Among known toxicants, V-series compounds tend to undergo perhaps the most varied transformations due to their unique structure. Possessing both high reactivity and polyfunctionality, these compounds incorporated into multicomponent matrices are capable of concurrently reacting with several components. Therewith, the reactions may involve different active centers within the same molecule. The routes and results of such reactions cannot be predicted in advance. The situation is even more complicated by the fact that active components of a natural or technogenic matrix are not always known. In view of this, research aimed at identifying transformation products of VX or VR in various media is of particular importance. The degradation of such compounds is suggested to be initiated by electron addition to phosphorus via reaction with anionic nucleophiles (Yang, 1999), such as hydroxide ions, water, alcohols, amines, or unsaturated organic compounds. The problem of determination of VR in complex matrices and identification of

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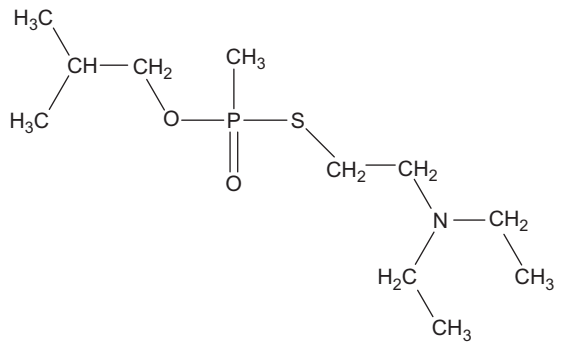


FIGURE 9.1 Structural formula of VR (Russian VX).

VR transformation products is still more complicated by the scarcity of available reference information.

9.2 Monitoring of Russian VX

A group of measures for assessing levels of toxic chemicals in the environment is termed *ambient monitoring*. The possible objects of ambient monitoring are air, water, soil, food, wastes, building materials, and other media through which toxicity factors can be delivered to the human organism. Biomonitoring is based on sampling and analysis of body fluids and tissues and is an indicator of internal dose that provides a measurement of exposure to a toxic chemical. It is quite understandable that toxicological studies are mainly focused on biomonitoring rather than on the levels of toxic compounds in environmental media. At the same time, ambient monitoring is the only tool for revealing sources of toxic pollutants affecting an organism.

9.2.1 Ambient monitoring of Russian VX

The composition of VX degradation products and admixtures has been studied in detail by hybrid chromatography–mass spectrometry (MS) methods. The objects of admixture studies were the contents of containers in which VX had been stored for a long time. Dozens of admixtures and stabilizers could be identified. There has been much work on the identification of admixtures in VX and its degradation products. A systematic review of VX transformation products is presented by Munro et al. (1999), but there are no such systematic data for VR. The degradation of VR in various media always produces complex mixtures of products that commonly contain dozens of both volatile and nonvolatile organic compounds. The prevalent volatile products are phosphorus-free alkyl diethylaminoethyl mono- and polysulfides. The electron impact (EI) mass spectra of these compounds are quite similar to each other and most commonly contain a single strong peak at *m/z* 86, formed by the $[(Et)_2NCH_2]^+$ ion. Among phosphorus-containing products, methylphosphonic acid (MPA) and its monoisobutyl and diisobutyl esters (iBuMPA and iBu_2MPA) are usually detected in certain quantities. These compounds are also present as admixtures in technical VR samples in varied amounts (from tenths of a percent to several percent). The P–S bond cleavage in VR forms diethylamino ethanethiol and iBuMPA. The latter slowly hydrolyzes to form MPA.

The most hazardous known VX hydrolysis products are persistent bis[2-(diisopropylamino)ethyl] disulfide, and highly toxic and persistent *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate. The decomposition of VR gives rise to structural analogs of the above products: bis [2-(diethylamino)ethyl] disulfide and *S*-[2-(diethylamino) ethyl] methylphosphonothioate. Systematic data on the behavior of these products in the environment, as well as on their acute and chronic toxicity to humans and mammals and ecotoxicity, are lacking. Components of the complex mixture of VR decomposition products present as low-informative EI mass spectra; therefore, these compounds are barely possible to identify solely on the basis of mass spectral data. Chemical-ionization (CI) mass spectra are much more characteristic, but CI mass spectra—in view of their irreproducibility and lack of databases—are of limited use for identification. Like VX, VR does not possess a strong electron-acceptor center favoring decomposition but still is not a complete analog of VX in this respect. In dilute aqueous solutions, VR proved to be much more persistent: a half-life of 12.4 days as opposed to 4.8 days for VX (Crenshaw et al., 2001). The mechanism of the neutralization of VX and VR with an equimolar amount of water was first described by Yang et al. (1996). It was found that autocatalytic hydrolysis is possible exclusively in V-series nerve agents since it should involve the protonated amino group.

We performed experimental research on the stability assessment of VR and identification of its transformation products under the action of equimolar or excess amounts of water. The method for analysis was gas chromatography–electron ionization mass spectrometry (GC-EIMS). In a dilute aqueous solution (10 mg/mL) in the presence of 5% phosphoric acid, the concentration of VR after exposure for 20 days at room temperature without stirring was 2.7 mg/mL, or 27% of the initial amount. Since among VR hydrolysis products both volatile and nonvolatile compounds could be expected, we chose three schemes for sample preparation: (A) evaporation to dryness followed by silylation with bis-(trimethylsilyl)trifluoroacetamide (BSTFA); (B) organic solvent extraction; and (C) silylation of the extract obtained by procedure (B).

Table 9.1 lists the principal products of VR hydrolysis with excess water in an acid medium, along with specified

TABLE 9.1 Products of VR hydrolysis with excess water in an acid medium.

RT (min)	Compound	Formula	Fraction	Content in the reaction mixture[a] (%[b])
6.709	*N,N*-Diethylformamide	$HCONEt_2$	**B**[c]	1
7.480	2-(Diethylamino)ethanethiol	$Et_2NCH_2CH_2SH$	**B**, C	10
7.917	*N,N*-Diethylacetamide	$MeCONEt_2$	**B**	<1
10.275	Methylphosphonic acid [as bis(trimethylsilyl) derivative]	$MePO[OSi(Me)_3]_2$	**A**, C	5
11.442	Isobutyl hydrogen methylphosphonate (as TMS derivative)	$CH_3PO(OiBu)OSi(Me)_3$	**A**, C	22
12.217	*O*-Isobutyl *S*-hydrogen methylphosphonothioate (as S-TMS derivative)	$CH_3Po(iBu)SSi(Me)_3$	A, **C**	11
12.674	Diisobutyl methylphosphonate	$MePO(iBuO)_2$	**B**, C	3
16.733	Bis(2-diethylaminoethyl) sulfide	$(Et_2NCH_2CH_2)_2S$	**B**, C	10
18.50	VR	$MePO(iBu)SCH_2CH_2NEt_2$	**A**, B, C	26
18,898	Bis(2-diethylaminoethyl) disulfide	$(Et_2NCH_2CH_2)_2S_2$	**B**, C	1

[a] *Hydrolysis time 20 days.*
[b] *Dozens of minor and insignificant components altogether amounted to near 10% and are not shown here.*
[c] *Bold-faced text is the fraction in which quantitative analysis for the component was performed.*

analytical fractions with the highest content of each compound. Fractions A, B, C listed in Table 9.1 are in correspondence with A, B, C schemes for sample preparation. It should be noted that even after 100-day exposure, about of 1% of the initial amount of VR in the solution was detected, which implied a uniform hydrolytic degradation.

Experiments on VR hydrolysis with an equimolar amount of water were performed as follows. A mixture of 74 μL of VR and 5.6 μL of water was exposed at room temperature for 3.5 months without stirring. An ash-gray thick homogenous material formed after hydrolysis and completely dissolved in 5 mL of acetonitrile. The solution was diluted 100 times with acetonitrile and analyzed by GC-EIMS (scheme B). An aliquot of this sample was mixed with an equal volume of BSTFA, and the mixture was heated at 70°C for 30 min and then analyzed by GC-EIMS (scheme C). The autocatalytic hydrolysis of VR was almost complete by the end of the experiment, since the VR content in the sample was no more than 0.01%. Qualitatively, the reaction mixture in the latter case was much poorer than in the hydrolysis with excess water. Among the volatile hydrolysis products, the following were detected by GC-EIMS: 2-(diethylamino)ethanethiol (4%), diisobutyl methylphosphonate (2%), bis[2-(diethylamino)ethyl] sulfide (2%), VR (<0.01%), and bis[2-(diethylamino)ethyl] disulfide (80%). Of nonvolatile products in sample, MPA (3%) and isobutyl ester of MPA (isobutyl MPA) (96%) were identified as trimethylsilyl ester. Isobutyl MPA, which catalyzes VR degradation, was detected as the major component of the reaction mixture. As the second most abundant component we expected 2-(diethylamino)ethanethiol, according to Yang et al. (1996), but we found instead that it was almost completely converted into bis[2-(diethylamino)ethyl] disulfide. This result seems feasible since once the autocatalytic degradation of VR was complete, nothing would prevent thiol from being converted into disulfide. In a dilute aqueous solution of VR, this conversion occurs less rapidly, and even after 100 days the solution contains much less [2-(diethylamino)ethanethiol than bis(2-diethylamino)ethyl] disulfide.

The safe levels of VR content on surfaces of various types and in the waste of building materials were established during the period of the chemical weapon destruction (CWD) program and were 2×10^{-6} mg/dm^2 for surfaces and 5×10^{-5} mg/kg for waste, respectively. In order to monitor compliance with these standards, in addition to biochemical methods, more selective gas chromatography techniques have been developed. Under standard GC analysis conditions, a direct determination of VR with a detection limit that meets hygiene standards is not achieved. Therefore, the conversion of VR to fluorohydride on a tablet impregnated with silver fluoride is used, followed by thermal desorption of fluorohydride into a gas chromatograph from a sorption tube. This approach was taken as the basis for the development of all official Russian methods for determining VR in swabs of the surfaces and in-depth samples by GC with mass

spectrometric, thermionic, flame-photometric, and pulsed flame-photometric detectors. VR was extracted from solid matrices by treating the ground material with a mixture of solvents in a Soxhlet apparatus followed by re-extraction of VR into acidified water (pH 4). The aqueous sample was alkalinized to pH 9 with borate buffer solution and VR was extracted into dichloromethane, followed by concentration of the extract to the required volume. When developing official methods, we focused on the use of gas chromatographs, since there were no HPLC instruments at the CWD facilities. In research laboratories equipped with modern instrumental techniques, including HPLC-MS/MS, methods for the direct determination of VX and VR, which excludes the stage of conversion to fluorohydride, were developed. Bell et al. (2001) reported on the directions of fragmentation when VX and VR were directly introduced into the electrospray ionization ion trap mass spectrometry (ESI/ITMS). It was found that in the electrospray ionization mode, different directions of fragmentation are characteristic for VX and VR. The main direction of fragmentation of the VX precursor ion is the loss of C_4H_8 alkene. For VR, a priori also expected loss of alkene, but it turned out that the predominant direction is the loss of the phosphorus-containing fragment. The peculiarity of VR fragmentation is apparently explained by the higher acidity of the proton at the quaternary carbon atom of the isobutyl group of VR compared to the methyl proton of the ethyl group of VX.

To determine VR and the main products of its conversion, the HPLC-MS/MS method was developed, implemented on a Shimadzu LC-20AD instrument equipped with an autosampler and a Shimadzu LCMS-8050 mass selective detector with electrospray ionization at atmospheric pressure. Within the framework of the complex procedure, the VR and four products of its conversion were simultaneously determined: *S*-2-(diethylaminoethyl) methylphosphonothioate, bis (2-diethylaminoethyl) disulfide, as well as mono- and di-isobutyl methylphosphonates. Paraoxon was used as an internal standard, a solution of which in acetonitrile was applied to a tampon in the case of washing analysis or added to a weighed sample of crushed material in the case of analysis of solid samples. Model samples of materials contaminated with VR were prepared in the laboratory. The analytes were extracted from tampons or crushed solid samples with methanol. The components were separated on a Gemini-NX 3μ C18 110A (Phenomenex) column 150×2 mm $\times 3$ μm in the gradient mode. Characteristics of the substances to be determined are presented in Table 9.2.

Detection was carried out in the mode of selected reaction monitoring (SRM, MS/MS): registration of product ions formed during fragmentation of the precursor ion during positive or negative ionization was carried out in accordance with the parameters presented in Table 9.3.

Mass spectrometric detection conditions:

- Mass spectrometric detector: Shimadzu 8050;
- Flow rate of the gas dryer: 10 dm^3/min;
- Auxiliary gas flow rate: 10 dm^3/min;
- Flow rate on the sprayer: 3 dm^3/min;
- Interface temperature: 300°C;
- Temperature of the desolvation line: −250°C;
- Heater temperature: 400°C;
- Voltage on the capillary: 3500 V.

Optimization of the parameters was carried out so that the maximum sensitivity was achieved for VR. When implementing the technique, the detection limits of VR make it possible to monitor its content on surfaces and in deep samples with limits of determination of 3.3×10^{-7} mg/dm^2 for surfaces and 4×10^{-5} mg/kg for the crushed solid samples. VR and/or *S*-2-(diethylaminoethyl)methylphosphonothioate identification in the comtaminated samples should be confirmed by repeated analysis of the sample and reference samples using either high-resolution HPLC-MS/MS with an UltiMate 3000 liquid chromatograph (Thermo, USA) and mass Thermo Scientific Q-Exactive selective detector with electrospray ionization at atmospheric pressure, or similar equipment, in accordance with the parameters presented in Table 9.4.

High selectivity of the technique ensures its unified application in the analysis of the environmental objects with various matrix compositions. In the course of model experiments, it was found that various materials are capable of irreversibly retaining VR and its conversion products to varying degrees. Thus, only free (extractable) forms of these compounds can be analytically determined.

9.2.2 Biomonitoring of Russian VX

The following biomarkers are usually considered in the biomonitoring of VR and toxicokinetic studies:

- Inhibited AChE and BChE of red blood cells and blood plasma;
- Free (intact) VR;
- Isobutyl methylphosphonic acid, a product of hydrolysis of VR;
- *O*-isobutyl methylfluorophosphonate, the reactivated product from the adducts with blood proteins under the action of an excess of fluoride ions;
- Phosphorylated serum albumin.

VR adducts with cholinesterase are not susceptible to rapid aging and, therefore, can be reactivated for a sufficiently long time, both spontaneously and induced by the action of reactivators (Koryagina et al., 2014). On the other hand, VR adducts with albumin are not susceptible to both aging and reactivation, so they are the most reliable biomarkers confirming exposure to this substance. Recently, the successful determination of VR adducts with albumin in dry blood plasma spots has been

TABLE 9.2 Formulas and molecular weights of analytes in the monitoring of laboratory samples contaminated with VR.

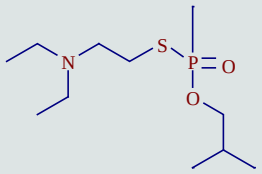
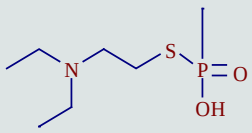
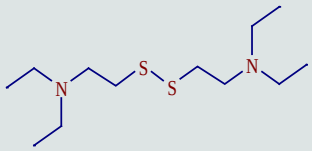
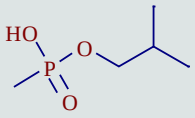
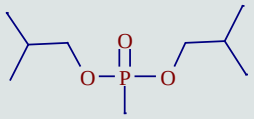
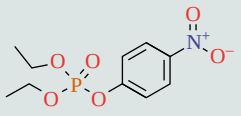
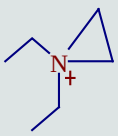
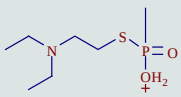
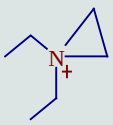
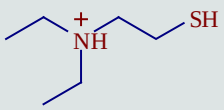
No.	Name	Structural formula	Gross formula	Molecular mass
1	*O*-Isobutyl-*S*-diethyl aminoethyl methylphosphonothioate (VR)		$C_{11}H_{26}NO_2PS$	267.14219
2	*S*-2-(Diethylaminoethyl) methylphosphonothioate		$C_7H_{18}NO_2PS$	211.07959
3	Bis(2-diethylaminoethyl) disulfide		$C_{12}H_{28}N_2S_2$	264.16939
4	*O*-Isobutylmethylphosphonic acid		$C_5H_{13}O_3P$	152.06023
5	Methylphosphonic acid diisobutyl ester		$C_9H_{21}O_3P$	208.12283
6	Paraoxon (internal standard)		$C_{10}H_{14}NO_6P$	275.055878

TABLE 9.3 Analytical characteristics of VR and its conversion products in the MRM (MS/MS) detection mode.

No.	Analyte	The mode of ionization	RT (min)	MRM transition
1	$C_{11}H_{26}NO_2PS$ (VR)	ESI (+)	3.4	268.00 > 100.10
				268.00 > 72.05
				268.00 > 212.00
2	$C_7H_{18}NO_2PS$	ESI (+)	1.3	212.10 > 100.10
				212.10 > 72.05
3	$C_{12}H_{28}N_2S_2$	ESI (+)	3.8	265.10 > 132.10
				265.10 > 86.15
4	$C_5H_{13}O_3P$	ESI (−)	3.3	151.05 > 95.05
				151.05 > 77.05
				151.05 > 79.05
5	$C_9H_{21}O_3P$	ESI (+)	4.9	209.00 > 96.95
				209.00 > 79.00
				209.00 > 47.00
6	$C_{10}H_{14}NO_6P$ (internal standard)	ESI (+)	4.6	276.00 > 220.00
				276.00 > 94.05

TABLE 9.4 Product ions of VR and *S*-2-(diethylaminoethyl) methylphosphonothioate using high-resolution HPLC-MS/MS equipment.

VR			*S*-2-(Diethylaminoethyl) methylphosphonothioate		
268.14946→100.11208		$C_6H_{14}N$	268.14946→212.08686		$C_7H_{19}NO_2PS$
212.08686→100.11208		$C_6H_{14}N$	212.08686→134.09980		$C_6H_{16}NS$

demonstrated (Koryagina et al., 2018). Using the HPLC-MS/MS method, rapid and reliable determination of the VR hydrolytic metabolite, isobutylmethylphosphonic acid, was achieved (Koryagina et al., 2016). The same as with other nerve agents, abundant excretion of isobutylmethylphosphonic acid with urine occurs during the first 2 days after a single exposure to VR, but in the next 2−3 weeks, detection of this urinary metabolite is still possible in order to establish the fact of exposure. Isobutylmethylphosphonic acid is usually found in the blood and tissues when renal excretion is impaired or discontinued. In postmortem analysis, after exposure to lethal doses, this biomarker is found in almost all bio-fluids and tissues of laboratory animals. Recently, the kinetics of alkyl methylphosphonic acids in plant tissues has been investigated (Sarvin et al., 2019). Garden grass was grown using hydroponics with solutions of alkyl methylphosphonic acids, including isobutyl methylphosphonic acid, a VR marker. After replacing the solution of alkyl methylphosphonates with pure water, alkyl methyl phosphonic acids could be detected in the roots and leaves for at least 5 weeks. Such a high retrospectiveness of the analysis was ensured by the stability of alkyl methylphosphonic acids in biomatrices, as well as by the high sensitivity of HPLC-MS/MS determination. Detection and identification of biomarkers when exposed to nonlethal doses of VR for significant intervals after exposure (up to several weeks) are implemented on the basis of modern chromatographic spectral technologies. Tandem mass spectrometry and high-resolution mass spectrometry proved to be the most effective analytical methods (Savel'eva et al., 2014).

The direct highly sensitive determination of V-agents in biological samples by HPLC-MS/MS made it possible to study the toxicokinetics of not only biomarkers (metabolites) formed in the body when exposed to V-agents (Reiter et al., 2008). In a number of studies, the possibility of direct highly sensitive determination of intact VR in blood plasma was confirmed, the lifetime of which in the body was longer than in VX (Cuquel et al., 2015; Reiter et al., 2015). When studying the molecular and stereoselective aspects of VX and VR in in vitro experiments, it turned out that the enantiomer (−)VR has a cholinesterase inhibition constant that is four times higher than the constant for (−)VX. VR showed a more pronounced stereoselectivity compared to VX. The ability to reactivate acetylcholinesterase inhibited by racemates or pure (−) enantiomers was found to be comparable for both agents. The differences in the toxicodynamic profiles of VX and VR are apparently caused by steric factors, in particular by the presence of the isobutyl group in VR. The toxicokinetics of the intact agent was studied at the molecular level by subcutaneous administration of VR to pigs. In this case, special attention was paid to the absorption mechanism. For cutaneous application of VX and VR at doses of $3 \times LD_{50}$, first-order absorption constants were determined for all enantiomers VX and VR. Four hours after exposure, significant differences were found in the rates of elimination of VX and VR; elimination of the latter being much slower. Furthermore, spontaneous reactivation of VR-inhibited AChE was observed to be faster than in the case of VX.

The expansion of the authority of the OPCW (the Organization for the Prohibition of Chemical Weapons) and the endowment of its attributive function, initiated studies aimed not only at identifying toxic substances but also at establishing their origin by identifying precursors and impurities, in order to establish the source of the prohibited substance. The first studies in this area were devoted to the attribution of VR (Holmgren et al., 2018; Jansson et al., 2018; Williams et al., 2018). In the cited works, the possibility of attribution is based mostly on statistical studies, though an extensive experimental material was obtained using the most advanced analytical technologies.

9.3 Mechanisms of action and principles of therapy

Three types of damage induced by OPs have been identified: acute poisoning; the so-called intermediate syndrome (IS); and OP-induced delayed polyneuropathy (OPIDP) (Ray and Richards, 2001). The IS symptoms and signs usually occur after apparent recovery from the acute cholinergic syndrome, but before OPIDP develops (Karalliedde et al., 2006). OPIDP also occurs almost exclusively in patients with preceding acute cholinergic toxicity related to severe acute exposure to an OP compound; neuropathy target esterase (NTE) is considered to be the principal molecular target for OPIDP (Lotti and Moretto, 2005). However, there are no data to indicate that VX has any potential at high or low doses for the induction of OPIDP in its classic manifestation in humans or other species, either with acute or long-term exposure. The reason could be that the ability of VX to inhibit NTE is nearly 1000-fold less than that of GB (Vranken et al., 1982; Gordon et al., 1983). Single i.m. injections of VX at 5 LD_{50} in atropine-protected chickens produced neither inhibition of NTE nor histological or behavioral evidence of OPIDP (Wilson et al., 1988a). There was no ability of VX at subchronic exposure (0.04 mg/kg for 90−100 days) to induce OPIDN in antidote-protected chickens (Wilson et al., 1988b). According to other data, NTE activity in brain areas and soleus muscles of rats was significantly depressed after they were subacutely exposed to VX in the absence of supporting therapy for 14 days at doses at the LD_{50} level and higher, and surviving animals exhibited muscle myopathy in the soleus muscles (Lenz et al., 1996). Blood AChE activity was depressed to zero throughout the experiment, so there is no contradiction on the lack of clear OPIDP signs under severe intoxication with VX.

9.3.1 Acute intoxication with Russian VX

A contributing factor to the high toxicity of VX may be its preferential reaction with AChE. Moreover, unlike the G agents, VX depresses AChE activity significantly more than BChE in humans (Sidell and Groff, 1974); the result is that more VX is available to react specifically with the target enzyme, AChE. In addition, indirect primary (connected with AChE inhibition) and secondary (not connected with AChE inhibition) effects of VR have also been described. Development of after-intoxication immunodeficient or immunotoxic states relate to the first group of effects (Germanchuk and Zabrodskii, 2005). In experiments with rats that were administered VR at a dose of 0.75 LD_{50}, a suppression of immune reactions was found: Th-1 cells significantly lost their functions and T-dependent immune reactions were depressed. In addition, an optimal balance of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in lymphocytes necessary for their proliferation and differentiation was disturbed (Zabrodskii et al., 2007). On the other hand, after a severe intoxication of laboratory rats with VR (2 LD_{50} with therapeutic treatment), there were no after-effects concerning the capability of the central nervous system (CNS) to produce conditioned reflex reactions, neither in the early post-intoxication period (2 weeks) nor at remote terms (1−6 months) (Novikova et al., 2007). Four different effects of RVX were ascribed to the second group (Prozorovskii and Chepur, 2001): (1) sensitization and desensitization of cholinoceptors to acetyl choline that is characteristic of M-cholinoceptors; (2) influence on acetyl choline release by nerve terminals: an inhibition after stimulation of M-cholinoceptors and facilitation after stimulation of N-cholinoceptors; (3) direct interaction with cholinoceptors, mainly with nicotinic ones, leading either to their activation or inactivation; and (4) interaction with ionic channels, mainly with that of N-cholinoceptors.

Hypoxic syndrome is one of the principal clinical manifestations under acute intoxication with VR, as well as other V-agents. It is triggered by disturbance of ventilation, which in its turn is caused by bronchospasm, bronchorrhea, convulsions, and central deregulation of respiration. A series of pathological mechanisms leads to reduction of circulating blood volume, decrease of blood vessel tone, and deregulation of vessel wall permeability. Abnormalities of blood rheology, caused by the loss of body fluids (salivation, bronchorrhea, etc.), and aggregation of blood cells significantly increase the dynamic viscosity of blood and aggravate disturbances of microcirculation. Disturbances of oxygen transport and its delivery to tissues induce secondary metabolic disorders and involvement in a pathological process of related biological systems that facilitates oxygen utilization by the tissues (Shestova and Sizova, 2005). An imbalance of electrolytes has also been described: in blood plasma under acute intoxication there was a decrease in sodium and potassium, the latter being more expressed than the former (Rybalko et al., 2005). Indirect secondary effects of OP agents, including VR, can be induced by excessive amounts of ACh in blood, with its action on cells having no cholinergic innervations. This can lead to deformation of red blood cells and endothelial cells, activation of basophils, and degranulation of mast cells (Prozorovskii and Chepur, 2001). In this context, one should keep in mind that endothelial cells have all the attributes of autonomic cholinergic regulation. Not only *M*-cholinoceptors but also *N*-cholinoceptors have been revealed (Hsu et al., 2005), as well as activity of AChE (Carvalho et al., 2005; Santos et al., 2007), the system of synthesis of acetylcholine (cholinacetyl transferase), and vesicular system of ACh transport out of the cells (Kirkpatrick et al., 2003).

9.3.2 Delayed effects: chronic and subchronic intoxication with Russian VX

The difficulty of diagnosis of delayed effects and chronic intoxication with VR relates to polymorphism of the clinical manifestations (Savateev et al., 2001). Long-term monitoring of personnel staff engaged in production of VR revealed slowly progressing signs of chronic intoxication (Gur'eva et al., 1997). The clinical signs of this may consist of functional and organic disorders of the CNS together with vegetative dysregulations, such as peripheral angiodystonic syndrome, vegetosensory polyneuropathy, or complex motor-sensory-vegetative pathology of axonopathic or myelinopathic type. Neuromuscular effects, visual and gastrointestinal disorders, immunodeficiency, and metabolic disorders have also been described (Gur'eva et al., 1997; Yanno et al., 2000). Cessation of contact with VR does not lead to involution of the clinical picture of chronic intoxication. Along with nervous, gastrointestinal, and motor disorders, there can be visual and cardiovascular diseases. Moreover, the chronic occupational pathology of the former workers of the facilities may develop in 3–6 years, even though they were exposed to no more than 10 times the maximum allowable concentrations of RVX and had no acute intoxications in their anamneses (Filippov et al., 2005). Taking into consideration various and mostly indefinite clinical manifestations of delayed and chronic effects of VR, an experimental search was undertaken to seek new possible mechanisms of the pathogenesis and novel functional signs of intoxication. To model chronic intoxication in experiments with animals, VR was dissolved daily in drinking water to concentrations of 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} g/100 mL. A group of five rats consumed 20 mL of VR aqueous solution daily. During the 3-month test, animals of the first group consumed daily VR with drinking water in a dose of 10^{-5} mg per 1 kg body weight (I), animals of the second group consumed a dose of 10^{-4} mg (II), and those of the third group consumed 10^{-3} mg (III). Measurement of AChE activity in red blood cells (RBC-AChE) was performed by Ellman's method, and the functional activity of platelets was investigated by a low-angle light-scattering technique that allows all stages of the platelet transformation to be assessed (Mindukshev et al., 2005). Investigation of monosynaptic myotatic reflex and conduction rate through the peripheral nerve fiber was conducted with *n. tibialis.* Comparative analysis of biochemical and physiological parameters studied is indicative of the complete absence of significant changes of RBC-AChE activity in rats of all three groups relative to the control, after exposure to the VR doses given above (not shown in this chapter). Conversely, platelets of test animals exposed to VR differed from the control by their pronounced instability, an indication of which was development of their spontaneous activation and aggregation (Fig. 9.2).

After 3 months of intoxication, two kinetic parameters of aggregation (namely: normalized maximal rate U_{max}; and effective concentration EC_{50}) were significantly increased in groups II and III. Two months after cessation of the chronic intoxication with VR (i.e., the rehabilitation period), significant differences of both kinetic parameters were found only for group III. Groups I and II showed significant increases of EC_{50}, but only a tendency ($P < .1$) to increase U_{max}. The final estimation of kinetic parameters of aggregation, made 6 months after rehabilitation, showed a significant decrease of U_{max} in group III and an increase of EC_{50} in all three intoxicated groups (Fig. 9.3). A significant increase of U_{max} suggests the sensitization of platelets with the primary activation of signaling ways via protein kinases, the action of which tends to increase the expression of GPIIb/IIIa receptors (Shattil et al., 1998). The increase of the EC_{50} parameter with further elevation of U_{max} points to a partial desensitization of $P2X_1$ and $P2Y_1$ receptors, as does the growing activity of the above-mentioned kinases.

Stimulation of the peripheral nerve trunk of intact animals leads to generation of muscle action potentials of three types. According to the duration of latent periods, they fall into the following order: (1) M-response (the result of the direct stimulation of α-motoneuron axons); (2) H-response (the monosynaptic response); and (3) polysynaptic responses with the variable latent period from 8–12 up to about 40 ms. In test animals of the III group, the changes of temporal parameters refer mainly to the latent period and duration of M-response (Table 9.5). Polysynaptic responses occur at all intensities of excitation and have a more pronounced character than in intact rats. A marked level and more distinct differentiation of the peaks of the complex action potential were noted.

The results obtained after examining the rats in group II differ from the control group more significantly. Along with normal action potentials (of the "spike" type), there were slow waves of depolarization of up to 30 ms duration. Another significant difference is the absence of the "subthreshold border"; that is, graduation of the increase of the amplitude of the action potential when the irritation stimulus enhances (Fig. 9.4). Such an event usually takes place in newborn animals and is caused by a slight differentiation of motor neurons (Bursian, 1983). The latent period of M-response, the rate of rising action potentials, and their duration increased significantly. The results of testing the rats of group I did not differ from the control. At the same time, some animals had the entire spectrum of pathologic reactions observed in rats of groups II and III: fasciculations, the presence of slow (local, depolarized) potentials, and paradoxical discharges. The opposite character of the changes in velocity of nerve impulse

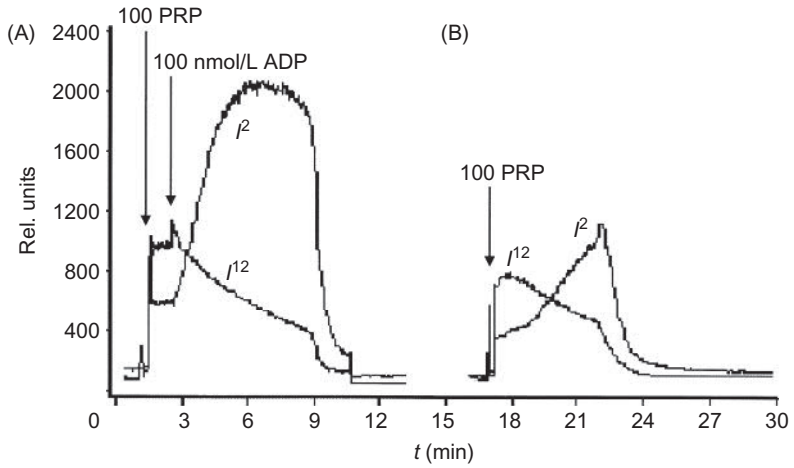


FIGURE 9.2 (A) ADP-induced activation and aggregation of blood platelets of control rats. (B) Spontaneous activation and aggregation of rat blood platelets immediately after 3 months of VR exposure at a dose of 10^{-4} mg/kg.
Inscriptions: *100 PRP* means 100 μL of *P*latelet-*R*ich *P*lasma; I^{12} and I^{2} indicate low angles of 12 and 2 degrees, respectively, at which the light scattering was recorded. *Reprinted from Mindukshev et al. Spectrosc. Int. J. 19, 247–257. Copyright (2005), with permission from IOS Press.*

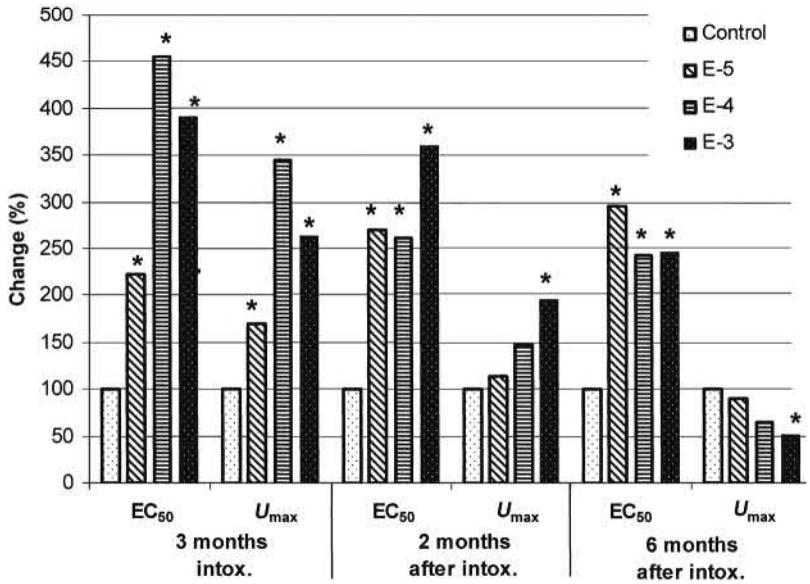


FIGURE 9.3 Kinetic parameters of rat platelet aggregation immediately after 3 months of VR exposure, and at 2 and 6 months after cessation of the intoxication (*$P < .05$ relative to control). E-5, E-4, and E-3 relate to doses of RVX (1×10^{-5}, 1×10^{-4}, and 1×10^{-3} mg/kg) consumed by rats daily with drinking water.
U_{max} means normalized maximum rate of platelet aggregation. *Reprinted from Mindukshev et al., Spectrosc. Int. J. 19, 247–257. Copyright (2005), with permission from IOS Press.*

conduction within groups II and III could be caused by different intensities of demyelinization of nerve fibers with different diameters.

9.3.3 Delayed effects: embryo- and gonadotoxicity, mutagenesis, and carcinogenesis

To study the embryotoxicity of VR, it was administered to pregnant female rats per orally at a dose of 1/100 LD_{50} (Kiryukhin et al., 2007). VR had a toxic effect in the females judged by a decrease in their RBC-AChE activity; at the same time, AChE activity in tissues of fetoplacental complex complied with age-specific control levels. These results may be regarded as evidence for impossibility (or at least low probability) of transplacental transfer of VR from the maternal organism to the fetus. However, manifestation of intoxication symptoms by pregnant rats indicates that VR can induce an embryotoxic effect, which is apparent from disorders of prenatal and postnatal ontogenesis (Tochilkina and Kiryukhin, 2007). It should be mentioned that embryotoxicity of VX to rat fetuses was also shown after single LD_{50} doses to the mother (Guittin, 1988), and after repeated doses of 0.005 mg/kg (near 1/5 LD_{50}) at varying times during fetal development (Guittin et al., 1987). On the other hand, no teratogenic potential of VX was found in sheep, rats, or rabbits (Van Kampen et al., 1970; Goldman et al., 1988).

Gonadotoxicity of VR was investigated in chronic experiments for different methods of introduction. Dermal exposure of male rats to VR demonstrated that general toxic effects were the governing factor of gonadotoxicity. There were no gonadotoxic effects at the level of threshold dose, which was estimated to be 4×10^{-6} mg/kg (Maslennikov and Kiryukhin, 2003). Other research has not revealed changes in the weight parameters of left and right testicles and epididymis, nor their total and specific weight parameters (Shabasheva et al., 2007). It has been concluded that VR poses no danger concerning the

TABLE 9.5 Electrophysiological parameters of the peripheral nervous system of rats after 3 months of VR chronic exposure.

	Control	I	II	III
t_M	1.36 ± 0.29	1.50 ± 0.19	1.72 ± 0.33[a]	1.07 ± 0.14[b]
$t_{peak\ M1}$	2.33 ± 0.54	2.79 ± 0.38	3.06 ± 0.42[a]	1.74 ± 0.44[b]
T_M	5.02 ± 1.87	6.73 ± 2.26	7.29 ± 1.70[a]	3.62 ± 0.96[b]
t_H	4.00 ± 0.41	3.51 ± 0.20	3.78 ± 0.20	4.02 ± 0.58
$t_{peak\ H1}$	4.60 ± 0.74	4.12 ± 0.33	3.95 ± 0.56[b]	4.35 ± 0.70

Symbols: t_M, M-response latent period; $t_{peak\ M1}$, latent period of the first maximum component of M-response; T_M, M-response duration; t_H, H-response latent period; $t_{peak\ H1}$, latent period of first maximum component of H-response.
[a]*Differences are significant at* $P < .01$.
[b]*Differences are significant at* $P < .05$.

development of specific disorders of male reproductive function, and this agrees with available data on VX: neither acute nor chronic VX exposure had deleterious effects on reproductive potential (Van Kampen et al., 1970). Studies of mutagenicity of VR in the Ames test revealed no point mutations in the indicative bacteria. However, studies conducted by a micronuclear test have demonstrated that a single intragastric introduction of VR at a dose of 1/10 LD_{50} stimulated in rats an enhancement of frequency of polychromatophilic erythrocytes with extranuclear inclusions. In cases where the cytotoxic effects were absent, these alterations could be interpreted as being of a mutagenic nature (Maslennikov and Ermilova, 2005). At the same time, the threshold dose of general toxic effect of VR had neither mutagenic nor embryotoxic nor gonadotoxic effects. Therefore, the delayed effects of VR can appear at doses exceeding those that induce general toxic effects, indicating the nonspecific character of its action. In the available literature, we could not find experimental data on the carcinogenicity of VR. Nevertheless, epidemiologic data on tumor and pretumor diseases of people that were engaged in production of VR did not reveal an increase in oncological morbidity compared to a control group of human subjects (Fedorchenko et al., 2003). In agreement with this, McNamara et al. (1973) reported that there was no association of increased cancer in personnel working daily with VX.

9.3.4 Principles of therapy

Acute toxicity of OPs in general and VR in particular has been much more extensively investigated than chronic toxicity, so it is not surprising that an effective therapy has been developed for acute intoxications only. The most effective antidote complex for treating acute VR intoxications consists of an antagonist of M-cholinoceptors, a reversible inhibitor of ChE, and a reactivator of ChE; in addition, anticonvulsants can be used in cases where convulsions occur. In experiments with guinea pigs, they were pretreated with pyridostigmine (0.026 mg/kg, i.m.), and then immediately after VR intoxication ($2 \times LD_{50}$) animals were given pralidoxime chloride (25 mg/kg, i.m.) and atropine sulfate (2, 8, or 16 mg/kg, i.m.); diazepam (5 mg/kg, i.m.) was administered to animals that displayed seizures and convulsions (Chang et al., 2002). It was also shown that in cases of acute intoxication with VR, higher doses of atropine should be administered compared to those for VX. Of several oximes (pralidoxime, obidoxime, and HI-6), the most effective was HI-6, as was the case for VX, sarin, cyclosarin, and soman poisonings (Kassa et al., 2006).

When developing therapies to treat chronic, low-dose VX intoxication, one should keep in mind that the VX-AChE complex has been found to undergo a significant degree of spontaneous reactivation in humans (at a rate of about 1%/h over the first 70 h after i.v. administration of VX). Another feature of VX toxicity is the lack of aging or stabilization of the VX-AChE complex and the relative ease of reactivation of VX-poisoned enzymes by oxime antidotes in humans (Sidell and Groff, 1974). Because there are no major differences in the reactivation process of VX- and VR-inhibited cholinesterase (Kuca et al., 2006), natural inactivation with plasma and liver paraoxonase (PON1) of VR, VX, and other warfare agents could play a greater role in prophylactic therapy of acute and chronic intoxications and their delayed effects (Costa et al., 2005).

9.4 Toxicometry and hygienic regulations

In countries dealing with destruction of CWAs, control limits for exposure via surface contact of drinking water are needed, as are detection methods for their low levels in water, soil, or foodstuffs. Some of the toxicity parameters of VR for humans and animals are available in the *Textbook of Instructive and Technical Documentation on*

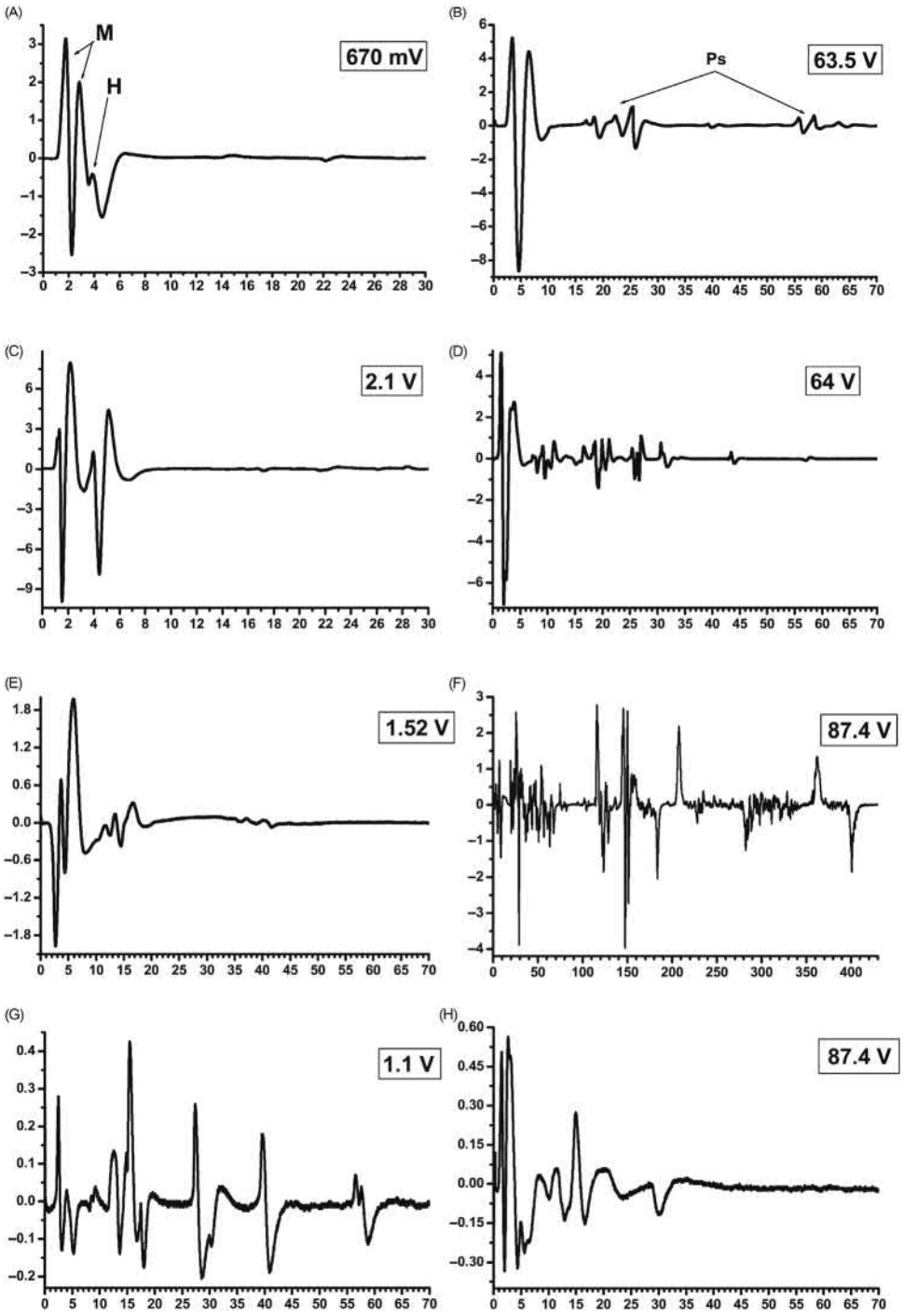


FIGURE 9.4 Action potentials of the rat gastrocnemius muscle under stimulation of *n. tibialis*: Effect of direct stimulation of the muscle (M-response, M), mono- (H-response, H), and polysynaptic (Ps) responses of control rats (A, B), rats of groups III (C, D), II (E, F), and I (G, H). Ciphers within frames indicate value of the stimulus. The *x*-axis shows the time of registration in ms; the *y*-axis shows the amplitude of action potential in mV.

the Problem of Chemical Weapon Destruction (Anon, 2001) and are given in Table 9.6.

In the event of possible contact of human skin with contaminated fabric of a protective suit, toxicometric parameters of VR have been experimentally estimated with laboratory rats (Zhukov et al., 2007): $LD_{50} = 0.55 \pm 0.09$ mg/kg, or 6.9×10^{-3} mg/cm^2; Lim ac (integr.) $= 0.056 \pm 0.001$ mg/kg, or 7.0×10^{-4} mg/cm^2; Lim ac (sp) $= 0.0051 \pm 0.001$ mg/kg, or 6.4×10^{-5} mg/cm^2; Lim ch $= 4.75 \times 10^{-5}$ mg/kg, or 5.9×10^{-7} mg/cm^2. The maximum concentration limit of VR for the fabric of protective suits has been estimated to be 3.1×10^{-8} mg/cm^2, taking into consideration the reserve coefficient, average body weight, and total area of the skin covered.

For the purposes of sanitary regulations, it has been estimated that VR within the range of concentrations

0.01–1.0 mg/L has no negative influence on the natural purification of aquatic reservoirs, on growth and decay of saprophytic and pathogenic microflora, and on nitrification processes. The noneffective dose of VR has been estimated to be 1×10^{-7} mg/kg, the threshold dose 1×10^{-6} mg/kg, and the effective dose 1×10^{-5} mg/kg (Maslennikov and Ermilova, 2006). Studies on VR effects on soil microflora have revealed that actinomyces and micromycetes proved to be the most vulnerable, whereas *Nitrobacteria* was the least vulnerable species (Gorbunova and Maximova, 2003). The hygienic regulations for VR are presented in Table 9.7.

9.5 Concluding remarks and future research

In this chapter, the principal products of VR hydrolysis with excess water in an acid medium have been presented. It has been shown that after 3 months of exposure, nearly 1% of the initial amount of VR in the solution could be detected. According to data on VR hydrolysis with an equimolar amount of water, the autocatalytic hydrolysis of VR was almost completed after 3 months since the VR content in the sample was no more than 0.01%. Moreover, the reaction mixture was much poorer than in the case of hydrolysis with excess water. Isobutyl MPA, which catalyzes VR degradation, was detected as the major component of the reaction mixture.

The extremely low recoveries of VR from materials of various natures can be explained by both irreversible sorption and degradation. A summarized list of compounds isolated from samples of building materials has been presented, which were taken from some areas of a former VR production facility and might have contacted VR. A brand-new approach is presented for the determination of VR and its conversion products in samples taken at former CWD facilities for possible reprofiling. The toxicological features of VR have not been fully clarified. On the one hand, pathogenesis of acute intoxication has been described, and toxicometric parameters and hygienic regulations have been developed. On the other hand, mechanisms of nonspecific effects, chronic intoxication, and the pathogenesis of delayed manifestations need further clarification. That is why there is a lack of effective prophylactic and therapeutic means of treating the delayed effects of VR. Our data support the existence of a key role for nonsynaptic mechanisms in developing effects under chronic exposure to VR. The morphofunctional changes at the level of the microcirculatory bed that influence the functional state of platelets may prove to be significant factors in the etiology of delayed effects of chronic intoxication. An adequate interpretation of the experimental data would lead to a proper understanding of the therapeutic approaches, which could prevent or even reverse the development of delayed effects of VR and other warfare OPs.

TABLE 9.6 Parameters of VR toxicity for animals and humans.

Parameter	Rout of exposure	Dose
LCt_{50} (human)	Inhalation	0.04 mg/min/dm^3
CL_{50} (mouse)	Inhalation	$1.8–4.5 \times 10^{-5}$ mg/kg
LCt_{50} (mouse)	Inhalation	0.011 mg/min/L
LD_{50} (rabbit)	Percutaneous	0.014 mg/kg
LD_{50} (cat)	Percutaneous	0.01 mg/kg
LD_{50} (dog)	Percutaneous	0.0157 mg/kg
LD_{50} (mice)	Percutaneous	0.016 mg/kg
LD_{50} (human)	Percutaneous	0.1–0.01 mg/kg

TABLE 9.7 VR safety standards in the Russian Federation (Uiba et al., 2007).

MAC for working air (mg/m^3)	5×10^{-6}
MAC for reservoir water (mg/dm^3)	2×10^{-6}
TSEL for ambient air (mg/m^3)	5×10^{-8}
MPL for equipment surface (mg/dm^2)	2×10^{-6}
MPL for human skin (mg/dm^2)	3×10^{-8}
MAC for soil (mg/kg)	5×10^{-5}
EEL for ambient air (mg/m^3)	
After 1 h	1.6×10^{-5}
After 4 h	4.1×10^{-6}
After 8 h	2.0×10^{-6}
After 24 h	6.6×10^{-7}

EEL, Emergency exposure limit; *MAC*, maximal allowable concentration; *MPL*, maximal permissible level of pollution; *TSEL*, tentative safety exposure level.

In conclusion, we outline some present and future areas of study on the analytical chemistry, biochemistry, and toxicology of VR:

- Improvement of the methodology of chemical and biochemical monitoring in environmental objects and human organisms;
- Development of prognostic modeling for probable risk assessment in case of elevation of the allowable levels of VR in various media;

- Studies on the quantitative relations dose−effect, time−effect, and dose−time−effect for cholinesterase and noncholinesterase effects, for both acute and chronic exposures to VR;
- Studies on novel molecular and functional effects under acute and chronic exposures to VR;
- Studies on mechanisms of development of delayed effects after intoxication with VR;
- Development of novel effective means for prophylaxis and treatment of delayed effects of intoxications with VR.

Fulfillment of these studies would surely contribute to fundamental and applied knowledge well beyond the toxicology of VR.

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Chapter 10

Novichoks

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10.1 Historical overview

After World War I, the idea of chemical warfare gained a firm position in the military doctrines of all developed countries around the world, without exception. Great Britain and France started with the improvement of known chemical warfare agents (CWAs) as well as an increase in the number of production capacities. According to the Versailles Treaty, defeated Germany was forbidden from possessing CWAs. Finally, the United States, having the most powerful chemical military potential, surpassed Great Britain and France in the production of toxic agents (Halámek and Kobliha, 2011; Klement, 2011).

In 1934, German chemical and pharmaceutical conglomerate I.G. Farben initiated a project focusing on synthetic insecticides. Otto Bayer was behind this development and handed over this research branch to Gerhard Schräder (Nepovimova and Kuca, 2018). In 1936, Schräder turned his attention toward organophosphorus (OP) compounds. This group of compounds had been well known since the beginning of the 20th century. However, Schräder's research opened the way for targeted synthesis of these biologically active substances. One of the first recognized OP compounds with a systemic effect was ethyl dimethylphosphoramidocyanidate, more commonly known as tabun (GA; Trilon, 83), synthesized by Lang and Krüger on December 23, 1936 (Klement, 2011; Szinicz, 2005). The success with tabun led to the establishment of a factory in Dyhernfurth (today, Brzeg Dólny, Poland). Of 3000 employees of this factory, several hundred were injured and several dozen died as a result of their employment. From 1942 to 1945, this plant produced up to 30,000 tons of tabun, of which 12,000−15,000 tons were filled into ammunition (Klement, 2011). In 1939, Schräder and his team found an OP compound that was similar to tabun—propan-2-yl methylphosphonofluoridate (GB; Trilon 46). This compound got its name from that of the team itself, that is, it was called sarin after Schräder, Ambros, Ritter, and Van der Linde (Coleman, 2005). After 1935, any invention that could in any capacity be used by the military had to be reported to the German Ministry of War, as per an official decree. In 1937, sarin and tabun were both sent to the German Army Weapons Office (Wa Prüf 9), and all patent applications that were related to such compounds were declared secret (Szinicz, 2005). A code name Trilon was created to carefully guard these compounds. After sarin and tabun were discovered, next in line was sarin's pinacolyl analogue, soman (GD; 3,3-dimethylbutan-2-yl methylphosphonofluoridate), discovered by Richard Kuhn and Konrad Henkel in 1944 (Tucker, 2006). The name was allegedly chosen from a Greek word that translates into "to sleep" (Kloske and Witkiewicz, 2019). All three of the compounds in question are essentially nerve agents (NAs) that fall under the collective title of G-agents, where G is an abbreviation for German. This is because they were all found by German researchers (Gupta, 2015).

Despite the mass production of G-agents by the Nazis, NAs have rarely been used on a mass scale in chemical warfare (Halámek and Kobliha, 2011). Additionally, the factory in Dyhernfurth even complicated the Yalta Conference in February 1945. Winston Churchill in his writings about World War II described how one of the last points in their discussion in Yalta was the border between Poland and Germany. According to the preliminary agreement, the border should have led along the river Oder and its tributary Neisse. However, by looking at map, it can be seen that Neisse has two tributaries—the Eastern and the Western. President Roosevelt suggested choosing the Eastern one, whereas Premier Stalin insisted on the Western. This disagreement delayed the negotiations for almost a day. Finally, the factory in Dyhernfurth fell to the Russians. Almost immediately after the end of

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WWII the factory was dismantled and all the reserves as well as the equipment were taken to the then Soviet Union and used for further development and production of toxic substances (Klement, 2011; Paxman and Harris, 2011; Pitschmann, 2014; Tucker, 2006).

As the Nazi regime was defeated, the Soviet and Allied forces—who were working together to defeat the Germans—took over NA production hubs. In addition, they also took over the research that the Germans had conducted on the subject. Thereafter, the Soviets as well as the Allies almost immediately started to develop and stockpile their own supplies of compounds (Wiener and Hoffman, 2004). In the 1950s, British chemist Ranajit Ghosh within his work on the development of novel organophosphorus (OP) pesticides, discovered a novel group of OP esters derived from thiocholine. Similar to G-agents, the existence of these compounds had to be reported to the British Chemical Warfare division due to their high toxicity and possible utilization by the military. The agent VX (*S*-{2-[di(propan-2-yl)amino]ethyl} *O*-ethyl methylphosphonothioate) has been labeled a promising agent within the set (Gupta, 2015; Szinicz, 2005). Within the military exchange program between the United States and the United Kingdom, the UK interchanged VX tech for thermonuclear weapons from the United States. Thereafter, large-scale synthesis of VX was launched in the 1960s in the United States (Tucker, 2006). At the same time, an isomer of VX—Russian VX (VR, RVX, substance 33, *S*-[2-(diethylamino)ethyl] *O*-(2-methylpropyl) methylphosphonothioate)—was created in the USSR. Later this compound became a prototype for A-agents. Another isomer of VX known as Chinese VX (CVX, *O*-butyl *S*-[2-(diethylamino)ethyl] methylphosphonothioate) was created and tested in a battery of experiments (Gupta, 2015). These are a class of NAs known as "V-agents." According to various sources, the *V* stands for "venomous," "victory," "virulent," or "viscous" (Gupta, 2015; Kloske and Witkiewicz, 2019).

During the Cold War, some long-term research programs were triggered in the USSR. Two of these, Fluorine and Phosphorus, were of particular interest in terms of not only the economy, but also in terms of the military sector. Special emphasis was placed on substances that had a significant biocidal impact (Halámek, 2008; Vásárhelyi and Földi, 2007).

At the same time, problems associated with the stability of Russian VX led to the start of a highly secretive program known as Foliant which ran from 1973 to 1976. The information acquired from the previous two programs was included in this new one. The idea behind it was to develop novel CWAs that would: (1) not be detectable through standard chemical detection tools being implemented by NATO member forces, (2) be able to penetrate the body of the enemy irrespective of what protective measures were in play, (3) be safer than previous iterations within the process of stockpiling, and (4) not be listed (also the precursors) in the Chemical Weapon Convention (CWC) (Vásárhelyi and Földi, 2007; Halámek, 2008; Halámek and Kobliha, 2011; Kloske and Witkiewicz, 2019). The endeavor associated with this program gave birth to a minimum of three unitary pieces of chemical weaponry, A-234, A-232, and A-230 (Fig. 10.1). Officially, the structures of the agents in questions have never found their way into scientific literature. Within the last couple of years, the data on these agents show that they are created from dihaloformaldoxime. The presumption is rooted in the scientific contributions of Soviet chemists that were likely a part of Foliant (Martynov et al., 1969; Petrov et al., 1967; Razumova et al., 1968; Kruglyak et al., 1972a,b; Malekin et al., 1972). It is interesting to note that a few decades before the discovery of A-agents the first acetylcholinesterase (AChE, E. C. 3.1.1.7) reactivators bearing an oxime functional group were published (Hobbiger, 1957). Later, however, it was disclosed that phosphorylated/phosphonylated oximes are formed as side products immediately after the reactivation process. These substances, instead of decreasing the toxic effect of OPs towards AChE activity strongly, inhibit the enzyme (Andersen, 1978; Fossier et al., 1983).

According to available data, "the A-agents faced the same issue as Russian VX," that is, low stability in the environment (Karev, 2009). To address this particular drawback, a binary form of A-agents was developed, passing the requirement of stability of their precursors. Finally, at least five precursors were developed under the code name Novichok (Karev, 2009). According to S.L. Hönig, the crucial moiety of Novichoks is 2-fluoro-1,3,2-dioxophospholane (Fig. 10.2) (Hoenig, 2007). The first mention of Novichoks appeared after the closing of the Foliant program (Petrov et al., 1967; Razumova et al., 1968; Martynov et al., 1969; Kruglyak et al., 1972a,b; Malekin et al., 1972; Mirzayanov, 2009).

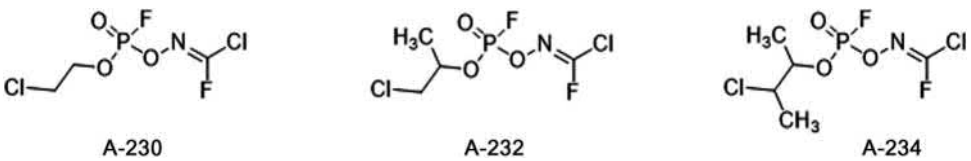

FIGURE 10.1 Possible chemical structures of A-agents (Nepovimova and Kuca, 2018).

In September 1992, a newspaper article revealing the existence of Novichoks was published on the eve of the ratification of the Chemical Weapon Convention by Russia (Mirzayanov, 1992). Vil Mirzayanov, an analytic chemist connected to the State Research Institute of Organic Chemistry and Technology authored the article. After the article was published, Vil Mirzayanov was arrested. However, due to an enormous public outcry, he was released after a couple of months. He then exiled himself to the United States (Halámek, 2008; Karev, 2009). Today, the data on the agents in question, alongside Novichoks, is still shrouded in mystery. The only available data come from the interviews and articles of Mirzayanov, Uglev, Zheleznyakov, and Fyodorov (Karev, 2009). The end of the Novichok program was probably associated with the ratification of CWC by the Russian Federation (Table 10.1) (Halámek, 2008).

10.2 Synthesis

The synthesis of compounds leading to the production of A-agents consists of three steps (Fig. 10.3) (Ellison, 2016; Halámek and Kobliha, 2011). The first two reactions of phosphorus trichloride with an appropriate diol and the subsequent nucleophilic substitution of a chlorine atom by fluorine should by all accounts lead to synthesis of the precursors to A-agents, known as Novichoks (Novichok ?, Novichok 5, Novichok 7). Finally, 2-fluoro-1,3,2-dioxaphospholanes react with dichloro(fluoro)nitrosomethane, a compound structurally resembling the choking agent, chloropicrin. According to S.L. Hoenig, the stability of formed products is temperature-dependent. At low temperature (−40°C), they are stable but when the temperature rises the nucleophilic attack by the chloride anion is facilitated, resulting in a phospholane ring opening (Hoenig, 2007; Halámek and Kobliha, 2011).

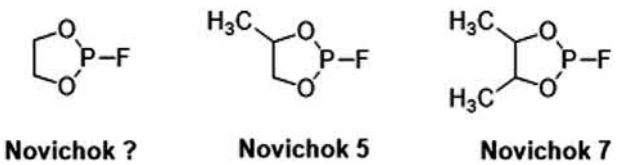

FIGURE 10.2 Plausible chemical structures of A-agent precursors—Novichoks (Nepovimova and Kuca, 2018).

From these data, the following findings are observed:

1. Although A-agents resemble fluorophosphates, they lack a C−P bond. Compounds without a C−P bond are out of CWC's scope.
2. The secondary alkoxy side chains, not methyl, ethyl, propyl, or isopropyl, are also outside the CWC scope.
3. CWC makes no mention of compounds with P−O−N linkage.
4. The precursor fluorodichloronitrosomethane does *not* fall under the regulation of CWC, even though its derivative chloropicrin does (Nepovimova and Kuca, 2018; "Organization for the Prohibition of Chemical Weapons," n.d.).

10.3 Physicochemical properties

Due to the scarcity of information and data pertaining to the physicochemical properties of A-agents and Novichoks, this section is presented in table form (Table 10.2) (Hoenig, 2007; Karev, 2009; Mirzayanov, 2009; Halámek and Kobliha, 2011; Pitschmann, 2014).

Considering the high volatility and low persistency of G-agents, as well as the low volatility and high persistency of V-agents, chemists from the military—who focused on third-generation NAs—looked at synthesizing compounds with properties that had a balance of the physical and chemical elements at play. This included aspects such as the density, volatility, and also stability in terms of moisture and light. As per the data outlined above, one can suppose that several drawbacks

TABLE 10.1 The outcomes of Foliant and then Novichok programs.

	RVX	RVX	A-230	A-232	A-232	A-234
Form	Unitary	Binary	Unitary	Unitary	Binary	Binary
Precursor	–	n.a.	–	–	Novichok 5	Novichok 7
Amount	15,000 tonnes	Tens of tonnes	Tens of tonnes	Several tonnes	Several tonnes	Tens of tonnes
Years of testing	n.a.	1988–9	1988–89	n.a.	1989–90	1993
Weaponization	n.a.	Weaponized in 1990	Weaponized in 1990	Was not weaponized	Approved in 1989	n.a.

n.a., Data not available.

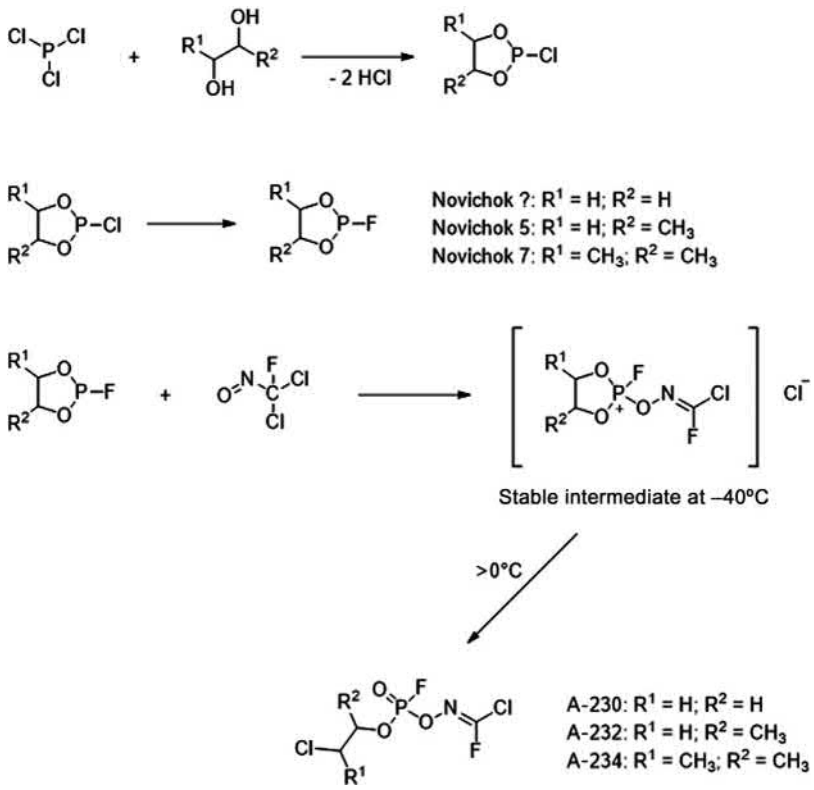

FIGURE 10.3 Possible synthetic route leading to A-agents (Nepovimova and Kuca, 2018).

TABLE 10.2 Physicochemical properties of A-agents.

	A-230	A-232	A-234
Molecular mass	241.95	255.97	270.00
Boiling point	61°C–62°C	70°C–71°C	73°C–74°C
Density	1.612 g/mL	1.515 g/mL	1.414 g/mL
State	Liquid	n.a.	Very viscous liquid
Behavior at low temperature	Solidifies at low temperature	Does not solidify at low temperature	n.a.
Volatility	Volatile	More volatile than A-230 or RVX	Poorly volatile
Moisture stability	Resistant to moisture	Less stable against moisture than A-230 or RVX	Resistant to moisture

n.a., Data not available.

characteristic of the first two generations of NAs were removed.

10.4 Mechanism of action

The mechanism of action of A-agents is irreversible inhibition of AChE (Hoenig, 2007). Once the A-agent reaches the bottom of the active site gorge, the nucleophilic attack of the phosphorus atom by the hydroxyl group of serine occurs (Mercey et al., 2012). This reaction is accompanied by a simultaneous cession of the fluoride ion and formation of phosphorylated enzyme (Fig. 10.4). The covalent bond shared by the phosphorus atom and serine constitutes a catalytic site of AChE, and substantially slows down spontaneous hydrolysis, varying from hours to days (Mercey et al., 2012).

Rapid hydrolysis of the $=N-O-$ bond within the A-agent–AChE complex may result in an aged form of

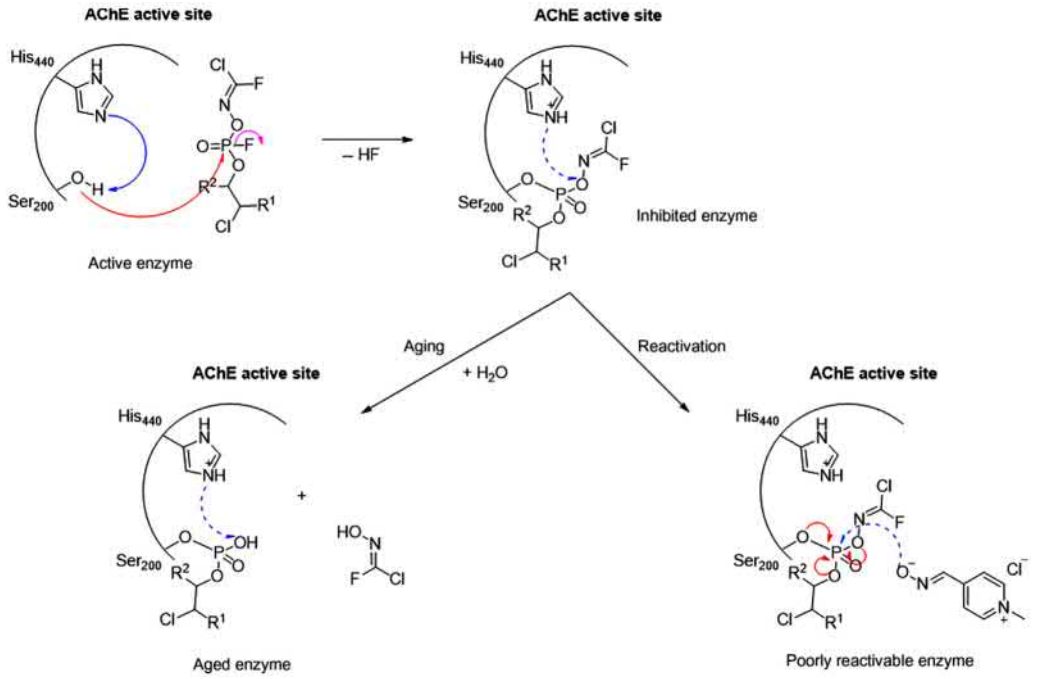

FIGURE 10.4 Mechanism of AChE inhibition by A-agents, aging, and reactivation by oximes (Nepovimova and Kuca, 2018).

TABLE 10.3 Toxicological parameters of G-, V-, and A-agents.

	LCt_{50} (mg min/m^3)	LD_{50} percutaneous administration (mg/person)
Sarin	100	1700
Soman	70	350
VX	50	10
A-232	6–10	1–2
A-234	7	5

AChE where the phosphonic oxyanion forms a salt bridge with the protonated histidine that strongly stabilizes the conjugate (Fig. 10.4). Aged enzyme is permanently inactivated and no reactivator can restore its function (Mercey et al., 2012; Sharma et al., 2015). According to the available data, the aging half-time of A-230 and soman are relatively similar (2–4 min) (Worek et al., 2004; Karev, 2009).

Reactivation of inhibited AChE by A-agents appears to be relatively difficult. This is true not just due to rapid aging, but also because of the phosphorus atom's debilitated partial positive charge, which is attacked by the oximate of the available reactivators (Fig. 10.4). Thus, the only effective treatment approaches seem to be symptomatic therapy (combination of an anticholinergic drug and an anticonvulsant) or administration of so-called bioscavengers, such as butyrylcholinesterase (BChE, E. C. 3.1.1.8). Such exogenous enzymes would detoxify A-agents entering the patient's circulation. Thereafter, toxic substances would not reach AChE and evoke clinical signs of intoxication (Bajgar et al., 2009). Generally, bioscavengers are considered a prophylactic approach. However, in the case of A-agents poisoning when causal therapy is ineffective, they can be used ex post. Additionally, bioscavengers should be able to detoxify all types of NAs, thus representing a universal approach.

10.5 Toxicity

Available sources claim that A-agents display a higher or the same level of toxicity as VX. Exact toxicological

parameters of A-agents, as well as of representatives of G- and V-agents, are summarized in Table 10.3.

If the victim survives the acute phase of intoxication by the A-agent, the delayed neurotoxicity marked by serious nerve system disruption manifesting as paralysis, taking place at 1−3 weeks subsequent to intoxication, could occur (Gupta, 2015). One of the scientists working on A-agents development, Andrey Zheleznyakov, suffered exposure to A232. He experienced multiple health issues including epilepsy, cirrhosis, and trigeminal neuritis and died 5 years after his exposure (Karev, 2009).

10.6 Concluding remarks and future directions

Although many years have passed since the disclosure of the Novichok program, exact reliable data do not exist. If even a small part of the information holds true, it is imperative that the risk that such lethal weapons holds be acknowledged. It is important to strengthen global verifications and regulations. The priority should be inclusion of A-agents, alongside their precursors, to the CWC. After this, a small amount of the agents should be synthesized to discern how efficient a possible antidote could be to deter its side effects and/or to create impactful detection strategies.

Acknowledgment

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Chapter 11

Blister agents

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11.1 Introduction

As their name implies, blister agents (also referred to as vesicants) cause blistering lesions in the skin (Anslow et al., 1947; Graef et al., 1948). The respiratory tract and eyes, however, are more sensitive targets of toxicity. Respiratory tract effects are the primary cause of acute lethality. The mode of action of blister agents is complex and continues to be a focal point for research. These cytotoxic alkylating agents were initially developed as chemical weapons used to induce ocular, dermal, and respiratory damage that results in immediate casualties, reduction in fighting efficiency, and demoralization. Depending on the exposure, injury may be local or systemic. This chapter focuses on sulfur mustards, nitrogen mustards, and lewisite. Although occasionally classified as a vesicant due to its action as a skin, eye, and respiratory tract irritant, phosgene oxime (CX) is more appropriately considered a urticant, or nettle agent, and is not discussed here. Extensive information regarding the chemistry and toxicology of vesicants is available in several publications (Papirmeister et al., 1991; Somani, 1992; USACHPPM, 1996; ATSDR, 2003; Romano et al., 2008). In response to a request by the US Department of Veterans Affairs (VA), a panel of experts extensively reviewed and evaluated the medical and scientific literature on mustard agents and lewisite, as well as the military testing programs relative to these agents (IOM, 1993).

Although generally associated with World War I, the Organization for the Prohibition of Chemical Weapons (OPCW) documented the use of sulfur mustard in Umm Hawsh, Syria, in 2016 (UNSC, 2017). Blister agents were also deployed by Iraq against Iranian forces and Kurdish citizens of Iraq during the Iran−Iraq war in the 1980s (Haines and Fox, 2014).

The term *sulfur mustard* may refer to distilled mustard [bis(2-chloroethyl)sulfide; HD, sesqui mustard (SM)], Levenstein mustard (H), agent HT [a mixture of HD and bis (2-chloroethylthioethyl)ether], or a sulfur mustard−lewisite mixture (HL). Distilled mustard (HD) is relatively pure (97%) bis(2-chloroethyl)sulfide and results from the vacuum distillation of HD. Generic references to sulfur mustard usually mean HD. Levenstein mustard (H) is a mixture of HD and sulfur impurities (generally in a 70:30 ratio), and, as its name implies, it was produced by the Levenstein process, which involves reacting ethylene with sulfur chloride. Agent H may contain sulfur impurities imparting a yellowish color and sweet, garlic-like odor.

Agent HT is generally a mixture of 60% HD and 40% bis(2-chloroethylthioethyl)ether (T), although this ratio may vary. Agent HL is a mixture of sulfur mustard (HD) and lewisite (L) that was developed for cold weather or high-altitude use due to its lower freezing point. SM is 1,2-bis(2-chloroethylthio) ethane and is considered a more potent vesicant than HD, but its very low vapor pressure limits its effectiveness as a warfare agent. This condition is remedied by combining it with HD to form agent HQ. The removal of one chlorine from sulfur mustard results in "half-mustard" (2-chloroethyl ethyl sulfide, or CEES), a monofunctional sulfur mustard analog. Although retaining some alkylating properties, half-mustard is not as highly regulated as sulfur mustard and is frequently used in sulfur mustard research. Most of the discussion of sulfur mustards in this chapter will be about distilled sulfur mustard (HD).

Nitrogen mustards include HN1 [bis(2-chloroethyl) ethylamine], HN2 (2,2′-dichloro-*N*-methyldiethylamine), and HN3 [tris(2-chloroethyl)amine hydrochloride]. As will be discussed later in this chapter, some of these were used in therapeutic arenas rather than in warfare.

Lewisite [L or L-1; dichloro(2-chlorovinyl) arsine] is an arsenical vesicant developed early in the 20th century. Lewisite occurs as *cis*- and *trans*-isomers, the typical ratio

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being 10:90. Several impurities, including bis(2-chlorovinyl)chloroarsine (L-2) and tris(2-chlorovinyl)arsine (L-3), are typically present. The chemical and physical properties of *cis*- and *trans*-isomers are similar.

11.1.1 Sulfur mustards

Sulfur mustards are capable of causing severe skin and eye damage at very low concentrations. Table 11.1 shows the chemical name, synonyms, identification codes, chemical formula, and structural formula for sulfur mustard.

Although frequently referred to as "mustard gas," the chemical is a liquid at normal ambient temperatures. Due to its oily consistency and low aqueous solubility, sulfur mustard is persistent in the environment. Information on the half-life of HD in air is unavailable. As previously noted, impurities may impart a garlic-like odor to sulfur mustard. Odor thresholds ranging from 0.15 to 0.6 mg/m^3 have been reported for sulfur mustard (Dudley and Wells, 1938; Bowden, 1943; Fuhr and Krakow, 1945; Ruth, 1986).

Watson and Griffin (1992) have summarized information on the distribution of unitary chemical weapon stockpiles in the United States. The chemical and physical properties of sulfur mustard (agent HD) are shown in Table 11.2.

The water solubility of sulfur mustard has been reported as 0.092 g/100 g water at 22°C (DA, 1974), and 5×10^{-3} M at room temperature (MacNaughton and Brewer, 1994). In dilute aqueous solutions, sulfur mustard hydrolyzes almost completely to thiodiglycol and hydrochloric acid (Papirmeister et al., 1991). For dissolved HD, the hydrolysis half-life ranges from about 4 to 15 min for temperatures of 20°C–25°C. Bulk HD may persist in water for up to several years (Small, 1984). It has been estimated that it takes 15 days for the mass of a 1-cm droplet of HD in quiescent water to decrease by half (Small, 1984). The Henry's law constant for HD has been estimated to be 2.1×10^{-5} atm m^3/mol (MacNaughton and Brewer, 1994), indicating a moderate potential for evaporation from water.

The persistence of sulfur mustard in soil depends on the soil type, pH, moisture content, and whether the agent is at the soil surface or buried. Small (1984) reported that HD applied to the soil surface volatilized and would likely be the main route of HD loss (with a half-life of about 30 min). In wet soil, however, hydrolysis would be the primary loss pathway. When sprayed onto soil, a vesicant action may persist for about 2 weeks, but when the agent continually leaks into the soil, vesicant action may be present after 3 years (DA, 1974). Rosenblatt et al. (1995) state that the persistence of sulfur mustard in soil is due to the formation of oligomeric degradation products that coat the surface of the mustard agent and that are resistant to hydrolysis. This may greatly enhance the environmental persistence of sulfur mustard. Sulfur mustard has a log K_{ow} of 1.37 and a K_{oc} of 133, indicating that binding to soil organics would limit transport through soil to groundwater (MacNaughton and Brewer, 1994). MacNaughton and Brewer (1994) also calculated a leaching index of 7.2 for HD (i.e., the number of leachings required to reduce the HD soil concentration to one-tenth of the original amount, assuming that for each leaching, 1 kg of soil is in equilibrium with 1 L of water).

11.1.2 Nitrogen mustards

Nitrogen mustards are tertiary bis(2-chloroethyl)amines with vesicant activity (NDRC, 1946). Tables 11.3 and 11.4 summarize the nomenclature and chemical and physical properties of HN1, HN2, and HN3. Due to their toxicity and various physical–chemical properties, initial interest in these chemicals as warfare agents developed shortly before and during World War II. Although HN2 and HN3 were specifically developed as military agents, HN1 was originally developed as a pharmaceutical. HN2 (mechlorethamine) later found use as an antineoplastic agent. Nitrogen mustards and derivatives such as melphalan, chlorambucil, and cyclophosphamide are alkylating agents used as cancer therapeutic agents (Somani, 1992).

TABLE 11.1 Nomenclature, chemical formulae, and chemical structure of sulfur mustard.

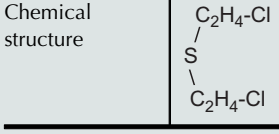
Sulfur mustard (HD)	
Synonyms	Bis(2-chloroethyl)sulfide; 1,1′-thiobis(2-chlorethane); 1-chloro-2-(2-chloroethylthio) ethane; SM; distilled mustard; agent HD; mustard gas; yperite; yellow cross
CAS No.	505-60-2
Chemical formula	$C_4H_8Cl_2S$
Chemical structure	

TABLE 11.2 Selected physical and chemical properties of sulfur mustard.

	Value	References
Physical state	Oily liquid	MacNaughton and Brewer (1994)
Molecular weight	HD: 159.08	DA (1996)
Density	5.4	DA (1996)
Boiling point	HD: 215°C–217°C	Budavari et al. (1989), DA (1996)
Freezing point	HD: 14.5°C	DA (1996)
Vapor pressure (mmHg)	HD: 0.072 mmHg at 20°C; 0.11 mmHg at 25°C	DA (1996)
Water solubility (g/L)	Sparingly soluble in water; soluble in organic solvents	Budavari et al. (1989), DA (1996)

TABLE 11.3 Nomenclature, chemical formulae, and chemical structures of nitrogen mustards.

HN-1	
Synonyms	Ethyl-bis(2-chloroethyl)amine; bis-(2-chloroethyl)ethylamine
CAS No.	538-078
Chemical formula	$(ClCH_2CH_2)_2NC_2H_5$
HN-2	
Synonyms	Methyl-bis(-chloroethyl)amine; 2,2′-dichloro-*N*-methyldiethylamine; "*S*"; mechlorethamine
CAS No.	51-75-2
Chemical formula	$(ClCH_2CH_2)_2NCH_3$
HN-3	
Synonyms	Tris(-chloroethyl)amine; [tris(2-chloroethyl)amine hydrochloride]
CAS No.	555-77-1
Chemical formula	$N(CH_2CH_2Cl)_3$

TABLE 11.4 Physical and chemical properties of nitrogen mustards.

	Value	References
Physical state	HN-1: oily liquid HN-2: oily liquid HN-3: oily liquid	USACHPPM (1996)
Molecular weight	HN-1: 170.08 HN-2: 156.07 HN-3: 204.54	USACHPPM (1996)
Boiling point[a]/freezing point	HN-1: 194°C/ − 34°C HN-2: 75°C/ − 60°C HN-3: 256°C/ − 3.7°C	USACHPPM (1996)
Vapor pressure (mmHg)	HN-1: 0.25 mm @ 25°C HN-2: 0.43 mm @ 25°C HN-3: 0.01 mm @ 25°C	USACHPPM (1996)
Water solubility (g/L)	HN-1: limited; miscible with organic solvents HN-2: limited; miscible with organic solvents HN-3: limited; miscible with organic solvents	USACHPPM (1996)

[a]*Decomposes prior to reaching boiling point.*

11.1.3 Lewisite

Lewisite is composed of *cis*- and *trans*-isomers in the ratio of 10:90 and several impurities including bis(2-chlorovinyl)chloroarsine (L-2) and tris(2-chlorovinyl) arsine (L-3) (Rosenblatt et al., 1975). The chemical and physical properties of the *cis*- and *trans*-isomers are similar. The nomenclature and chemical and physical properties of L-1, L-2, and L-3 are presented in Tables 11.5 and 11.6, respectively. In pure form, lewisite is colorless and odorless, but it usually occurs as a brown oily liquid with a distinct geranium-like odor. Gates et al. (1946) reported an odor threshold of 14–23 mg/m^3 for lewisite.

Information regarding the atmospheric transformation of lewisite is limited. MacNaughton and Brewer (1994) reported that some photodegradation may take place and that hydrolysis may also occur in the gas phase. Lewisite is only sparingly soluble in water; 0.5 g/L (Rosenblatt et al., 1975). Hydrolysis of lewisite results in the formation of lewisite oxide and HCl, and this may occur rapidly. The hydrolysis of lewisite is complex and includes several reversible reactions (Epstein, 1956; Rosenblatt et al., 1975; Clark, 1989; MacNaughton and Brewer, 1994). Under slightly acidic conditions, lewisite initially undergoes rapid and reversible conversion to dihydroxy arsine, 2-chlorovinyl arsine oxide, and two equivalents of hydrogen chloride:

$$Cl-CH=CH-AsCl_2 + 2H_2O \rightarrow Cl-CH=CH-AsCl_2 + 2HCl$$

The production of two equivalents of chloride occurs within 3 min at 20°C; at 5°C, the reaction is 90% complete within 2 min, and completion of the reaction requires several hours. The hydrolysis rate constant is reported as 1 min^{-1} at 20°C. Hydrolysis of 2-chlorovinyl arsine oxide is slower, resulting in lewisite oxide (chlorovinyl arsenous oxide) and polymerized lewisite oxide:

$$Cl-CH=CH-As(OH)_2 + H_2O \rightarrow Cl-CH=CH-AsO + (Cl-CH=CH-AsO)_n$$

The forward reaction is favored because lewisite oxide and polymerized lewisite oxide are insoluble. In a basic

TABLE 11.5 Nomenclature, chemical formulae, and chemical structures of lewisite.

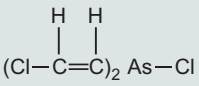
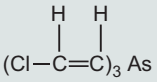
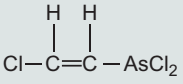
Lewisite (L)	
Synonyms	2-Chlorovinyldichloroarsine; (2-chlorovinyl)arsenous dichloride; beta-chlorovinyldichloroarsine; dichloro(2-chlorovinyl) arsine; chlorovinylarsine dichloride; EA 1034
CAS No.	541-25-3
Chemical formula	$ClCH{=}CHAsCl_2$
Chemical structure	
L-2	
Synonym	Lewisite-2
CAS No.	40334-69-8
Chemical formula	$(ClCH{=}CH)_2AsCl$
Chemical structure	
L-3	
Synonym	Lewisite-3
CAS No.	40334-70-1
Chemical formula	$(ClCH{=}CH)_3As$
Chemical structure	

TABLE 11.6 Physical and chemical properties of lewisite.

	Value	References
Lewisite		
Physical state	Oily, amber brown liquid	Lindberg et al. (1997)
Molecular weight	170.08	USACHPPM (1996)
Boiling point	190°C	Trammell (1992)
Freezing point	−18°C; varies depending on purity	Watson and Griffin (1992)
Vapor pressure (mmHg)	0.34 mmHg at 25°C; 0.22 mmHg at 20°C	USACHPPM (1996)
Water solubility (g/L)	0.5 g/L in water; soluble in most organic solvents	USACHPPM (1996)

solution, the *trans*-lewisite isomer is cleaved by the hydroxyl ion to give acetylene and sodium arsenite; this reaction may occur even at low temperatures (Rosenblatt et al., 1975; Clark, 1989). *Cis*-lewisite heated to over 40°C reacts with sodium hydroxide to yield vinyl chloride, sodium arsenite, and acetylene (Rosenblatt et al., 1975). In aqueous solution, the *cis*-isomer undergoes a photoconversion to the *trans*-isomer (Rosenblatt et al., 1975). Epstein (1956) reported that the toxic trivalent arsenic of lewisite oxide in standing water is converted to the less toxic pentavalent arsenic.

Lewisite in soil may rapidly volatilize or may be converted to lewisite oxide due to moisture in the soil (Rosenblatt et al., 1975). The low water solubility suggests intermediate persistence in moist soil (Watson and Griffin, 1992). Both lewisite and lewisite oxide may be slowly oxidized to 2-chlorovinylarsonic acid (Rosenblatt et al., 1975). Possible pathways of microbial degradation in soil include epoxidation of the C═C bond and reductive dehalogenation and dehydrohalogenation (Morrill et al., 1985). Due to the epoxy bond and arsine group, toxic metabolites may result. Additionally, residual hydrolysis may result in arsenic compounds. Lewisite is not likely to bioaccumulate. However, the arsenic degradation products may bioaccumulate (Rosenblatt et al., 1975).

11.2 History and background

11.2.1 Sulfur mustards

Sulfur mustard was synthesized and its biological activities first characterized in the 1800s (Guthrie, 1860; Niemann, 1860; Despretz, 1992). By the early 1900s, its synthesis was further refined and its use as a warfare agent had been established when used as Levenstein mustard during World War I. More recent use occurred during conflicts in the Middle East. Its oily nature makes it persistent on surfaces it makes contact with. Because sulfur mustard exerts toxic effects following dermal, ocular, and inhalation exposure, its use necessitated full body protection which, in turn, required the development of protective clothing and significant changes in warfare operations.

Minute quantities of sulfur mustard are used by various military and contract laboratories for defense research purposes, and for verification of Chemical Weapons Convention compliance. Bulk quantities of sulfur mustard are no longer manufactured in the United States. Military stockpiles of sulfur mustard are awaiting destruction or are in the process of being destroyed. Some sulfur mustard may also be found buried or abandoned at former defense sites. Sulfur mustard was frequently loaded into artillery shells and aerial bombs (often with lewisite). Various quantities of sulfur mustard also exist in other countries. Large amounts of sulfur mustard have been disposed of at sea.

Outside of military conflicts, exposure to sulfur mustard has occurred or may occur in work environments that involve chemical weapon materials (e.g., storage depots, demilitarization facilities, and research laboratories), during emergency response operations or remediation and decontamination activities, or during treaty verification activities in support of the Chemical Weapons Convention. Chemical weapons such as vesicants are still considered potential military threats and terrorist targets. The most likely route of exposure to sulfur mustard is via aerosol/vapor exposure of the skin, eyes, and respiratory tract.

11.2.2 Nitrogen mustards

Due to the toxicity and various physical−chemical properties of nitrogen mustards (which are structurally similar to sulfur mustard), initial interest in these chemicals as warfare agents began shortly before and during World War II. Like sulfur mustard, all are alkylating agents. This chapter only discusses the nitrogen mustards referred

to as HN1, HN2, and HN3; selected chemical and physical properties of these substances are summarized in Tables 11.3 and 11.4. Although HN2 and HN3 were initially investigated as military agents, HN1 was originally developed as a pharmaceutical. HN2 (mechlorethamine) later found application as a pharmaceutical. Nitrogen mustards and derivatives such as melphalan, chlorambucil, and cyclophosphamide are alkylating agents used as cancer therapeutic agents (Somani, 1992). HN1 and HN3 are among the chemical agents found in Chemical Agent Identification Sets (CAIS), which are considered a component of nonstockpiled material. Generally, nitrogen mustards have not had the interest or high profile that sulfur mustard and lewisite have.

11.2.3 Lewisite

Lewisite, an organoarsenic compound, was developed in an attempt to create a more effective blister agent than sulfur mustard. Its development is generally credited to Winford Lewis at the Catholic University, Washington, DC, and is based upon a thesis by Julius Nieuwland, who described the synthesis of lewisite from arsenic trichloride, acetylene, hydrochloric acid, and mercuric chloride. Early on, the compound was frequently referred to by the vividly descriptive term "Dew of Death." Like sulfur mustard, it is both a vesicant and a systemic poison with target tissues not limited to the skin. In an attempt to develop an antidote to lewisite, British anti-lewisite (BAL), also known as dimercaprol, was developed (Peters et al., 1945), which later became invaluable in the treatment of arsenic poisoning. Late in World War I and into World War II, large quantities of lewisite were manufactured by Germany, the United States, Italy, the Soviet Union, and Japan (reviewed by Trammell, 1992). Large amounts of lewisite were manufactured (up to 2 tons/day by Japan) and stored prior to and during World War II (Tanaka, 1988; Trammell, 1992). Lewisite was frequently a component (often mixed with sulfur mustard) of artillery shells and aerial bombs (often mixed with sulfur mustard). Like sulfur mustard, there are reports of large amounts of the compound being disposed of at sea (Spiers, 1968). With the possible exception of its use against Iranian soldiers during the Iran–Iraq conflict (Perera, 1985), there has been little or no use of lewisite in battle. Goldman and Dacre (1989) have reviewed the chemistry and toxicology of lewisite.

11.3 Toxicokinetics

11.3.1 Sulfur mustards

Due to its lipophilicity, toxicologically relevant amounts of sulfur mustard are absorbed in epithelial tissue (Papirmeister et al., 1991). Dermal absorption depends on the thickness of the epidermis and on the presence of moisture, which enhances penetration. Absorption tends to be greater at the base of hair shafts and in the hair follicle, where the epithelial tissue is thinner than the surrounding surface area (Papirmeister et al., 1991). Approximately 20% of sulfur mustard applied to skin may be rapidly absorbed, while 12%–50% of this may react and remain at the application site (Somani and Babu, 1989). About 12% of the absorbed material remains at the contact site; the rest enters the circulation (Renshaw, 1947). Renshaw (1947) noted that the rate of penetration is 1–4 $\mu g/cm^2$/min at 75°F.

For dermal exposure, penetration rates over 2–8 h ranged from 2.9% to 6.7% and rates of absorption ranged from 1.2% to 4.0% following application of 400 μg of radiolabeled sulfur mustard per cm^2 of isolated perfused porcine skin (Riviere et al., 1995). The average total recovery of the radiolabel was 9.3% (3.8%–17.7%), suggesting substantial loss due to volatilization.

Contrary to dermal absorption, little is known about absorption in the respiratory tract. Cameron et al. (1946) calculated the absorption of sulfur mustard vapor in the noses of rabbits and rhesus monkeys. The concentration of the agent in the nasal passages was 10%–30% of the chamber concentrations (40, 100, and 500 mg/m^3), implying an absorption of approximately 70%–90%.

Several studies using radiolabeled sulfur mustard have shown that sulfur mustard and its metabolites may be widely distributed in the body after percutaneous or intravenous exposure. Maximum levels of radioactivity were detected in the kidneys, lungs, and livers of rabbits following intravenous administration (Boursnell et al., 1946). At 15 min following percutaneous exposure of rats, sulfur mustard-derived radioactivity was found in all examined tissues except the eyes (Young et al., 1944). Similarly, Clemedson et al. (1963) noted uniform distribution of radioactivity in mice after either percutaneous or intravenous exposures, with most radioactivity occurring in the nasal region, kidneys, liver, and intestine. Hambrook et al. (1993) reported on the uptake and distribution of radiolabeled sulfur mustard in the skin and blood of rats after cutaneous application. It was found that much of the agent entering the blood binds to hemoglobin and, to some extent, with glutathione (GSH). Results of studies with rabbits showed that sulfur mustard was concentrated in the cornea and, to a lesser extent, in the iris, lens, and conjunctiva within 5 min after application (Axelrod and Hamilton, 1947).

The biotransformation of sulfur mustard after intravenous or intraperitoneal injection of radiolabeled compound in rats has been examined. Following intravenous injection, the major urinary metabolite was GSH-bis-chloroethyl sulfide conjugates (45% of total urinary radioactivity) and smaller amounts of sulfone conjugates (7%)

and thiodiglycol and its conjugates (14.4%) (Davison et al., 1961). Roberts and Warwick (1963) found the major urinary product of cysteine-bis-(β-chloroethyl)sulfone after intraperitoneal injection of sulfur mustard in rats. Papirmeister et al. (1991) concluded that hydrolysis to thiodiglycol and reaction with GSH are the most important routes of detoxification. This is supported by human data showing that thiodiglycol is present in the urine for 1 week or more after exposure (Wils, 1987).

11.3.2 Nitrogen mustards

The effects of time, temperature, and humidity on the vapor penetration of HN1 and HN3 into the forearm skin of human male volunteers were reported by NDRC (1945). Results of this research showed similar effects of temperature and humidity as observed for sulfur mustard (e.g., greater absorption with increased temperature and humidity). The penetration of HN1 and HN3 was found to be linear with time (5−20 min for HN1 and 30−60 min for HN3). At 71°F−72°F and 50%−52% relative humidity, the HN1 penetration rate was 2.8 μg/cm^2/min; for HN3, it was 0.18 μg/cm^2/min at 72°F−73°F and 45%−48% relative humidity. At 86°F−87°F and 47%−49% relative humidity, the HN1 penetration rate increased to 5.2 μg/cm^2/min, and the HN3 penetration rate increased to 0.3 μg/cm^2/min at 85°F and 47%−48% relative humidity. Excretion via the urine is likely a major route of elimination, especially due to the water solubility of the immonium ion (see Section 11.4).

11.3.3 Lewisite

Little information is available regarding the toxicokinetics of lewisite. It is readily absorbed by mucous membranes and, because of its lipophilicity, dermal absorption is significant (HSDB, 2019). Dermal absorption reportedly occurs faster for lewisite than for sulfur mustard (Hurst et al., 2008). Axelrod and Hamilton (1947) reported that radiolabeled (^{74}As) lewisite applied to a 0.43 cm^2 area of human skin was primarily fixed on the epidermis and that very little was found in the dermis; most was detected in hair and hair follicles. In experiments with guinea pigs, histological examination revealed that lewisite applied to skin entered the epidermis within 2 min and penetrated the dermis within 10 min (Ferguson and Silver, 1947). Only trace amounts were detectable in the dermis at 24 h postapplication.

11.4 Mode of action

11.4.1 Sulfur mustards

Sulfur mustard is a lipophilic bifunctional alkylating agent that efficiently enters tissue upon contact. The mode of action of sulfur mustard is multifaceted and complex, and has been reviewed by Papirmeister et al. (1991), Hurst et al. (2008), Smith et al. (2008), Shakarjian et al. (2010), and Ghanei and Harandi (2011). Efforts to understand the mechanisms of sulfur mustard toxicity are ongoing. Basically, sulfur mustard disrupts the interface of the epidermis and basement membrane causing blistering between the epidermis and dermis. Both immediate (immediate cell membrane damage) and delayed phases [secondary effects resulting from inflammatory responses, deoxyribonucleic acid (DNA) damage, and vascular leakage] have been described for sulfur mustard-induced dermal effects (Somani and Babu, 1989). Sulfur mustard may initiate intramolecular and intermolecular cross-links that provide many targets, including proteins, nucleic acids, and lipids. Many of the toxic effects of sulfur mustard also appear to be attributed to oxidative stress resulting from the disruption of normal cellular metabolism.

The sulfur mustard toxidrome is likely the result of multiple mechanisms including: (1) thiol depletion leading to intracellular calcium imbalance and subsequent cell death, (2) the alkylation of DNA and other cellular macromolecules resulting in necrosis and apoptosis, and (3) sulfur mustard-induced glutathione depletion and subsequent lipid peroxidation; and induction of inflammatory responses.

A key component of sulfur mustard toxicity is the formation of a sulfonium ion and resulting episulfonium intermediate that may react with sulfhydryl-containing macromolecules. Damage may include Ca^{2+} translocases (Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase), which depend on thiol groups to maintain cellular Ca^{2+} homeostasis, and microfilamentous proteins. The resulting increase in intracellular calcium levels ultimately causes a decrease in cellular integrity and induction of apoptosis. Oxidative stress in sulfur mustard toxicity has been reviewed by Smith et al. (2008).

The role of DNA alkylation and the poly(ADP-ribose) polymerase (PARP) hypothesis theory for sulfur mustard toxicity has been reviewed by Papirmeister et al. (1991) and Debiak et al. (2001). In this mechanism, DNA is the initial target of the mustard agent. Alkylated DNA purines are enzymatically depurinated, creating apurinic sites that are cleaved by apurinic endonucleases, resulting in DNA strand breaks. The accumulation of DNA breaks leads to activation of the chromosomal enzyme PARP, which, utilizing NAD^+, causes a severe reduction in cellular NAD^+. Depletion of NAD^+ results in the inhibition of glycolysis, and stimulation of the nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent hexose monophosphate shunt, ultimately resulting in the induction and secretion of proteases and subsequent cellular changes. Kehe et al. (2009) have reviewed dermal toxicity mechanisms of sulfur mustard. Sulfur mustard-induced apoptosis and activation of inflammatory mediators such

as various interleukins (ILs) such as IL-1alpha, IL-1beta, IL-6, IL-8, as well as tumor necrosis factors, also appear to be involved in the toxic response to sulfur mustard.

Papirmeister et al. (1991) noted that sulfur mustard-induced cytotoxicity is dose-dependent and that DNA appears to be more sensitive to mustard-induced alkylation than are other cellular constituents. The low-dose effects of sulfur mustard are characterized by genotoxicity and inhibition of mitosis. The loss of cellular reproduction may be due to bifunctional alkylation that ultimately prevents normal DNA replication. It was hypothesized that monofunctional DNA damage might be responsible for low-dose mutagenic and possibly carcinogenic effects.

Sulfur mustard-induced lipid peroxidation is a function of GSH depletion. For this mechanism, depletion of GSH results in an accumulation of reactive oxygen species via hydrogen peroxide-dependent processes (Miccadei et al., 1988). The oxygen radicals react with membrane phospholipids, forming lipid peroxides that alter membrane structure, causing it to break down.

Additional work has focused on the identification of possible biomarkers of sulfur mustard exposure and injury (Buxton et al., 2000, 2001; Danne et al., 2000). More recently, the role of metalloproteinases and collagen degradation (Gerecke et al., 2005), platelet-activating factor (Clark et al., 2005, 2006), and interaction with cytochrome P450 processes (Brimfield and Hodgson, 2005; Brimfield et al., 2006; Mancheco and Brimfield, 2006) is being investigated relative to the mechanism of action of sulfur mustard.

11.4.2 Nitrogen mustards

Similar to sulfur mustard, a key component of nitrogen mustard toxicity is the formation of a cyclic onium cation. This occurs in the presence of polar solvents such as water (Somani, 1992). The immonium ion may react with nucleophiles (such as nitrogen) in the base components of nucleic acids and with sulfhydryl groups in proteins and peptides. The precise mechanism of nitrogen mustard activity is unclear, but several have been proposed: DNA/RNA alkylation and resultant effects, effects on GSH, membrane effects (protein cross-linking, ion transport effects), and cytoplasmic effects (release of lysosomal enzymes). The possible mechanisms of nitrogen mustard have been reviewed by Gray (1989). Results of preliminary work by Elsayed and Omaye (2006) in mice given HN2 intraperitoneally showed pulmonary alterations indicative of oxidative stress and impaired detoxification processes that are consistent with the aforementioned mechanisms.

11.4.3 Lewisite

Dermal or intravenous exposure to lewisite leads to local skin edema and pulmonary edema due to increased capillary permeability. The increased capillary permeability results in blood plasma loss and resultant physiological responses collectively referred to as *lewisite shock*. Lewisite shock may be likened to shock observed in severe burn cases. It has been hypothesized that functional changes in the lungs, kidneys, respiratory tract, and cardiovascular and lymphatic systems may be the result of a disturbance of osmotic equilibrium (Goldman and Dacre, 1989).

Lewisite-induced vesicant and systemic toxicity are likely due in part to interactions with thiol groups (Goldman and Dacre, 1989). The interaction with enzyme sulfhydryl groups may cause inhibition of enzyme by the formation of stable cyclic structures with arsenic. These thiol interactions result in energy depletion, leading to cell death (Young, 1999).

To investigate the mechanism of lewisite-induced ocular effects, Tewari-Singh et al. (2016, 2017) evaluated the pathophysiology of corneal injury in New Zealand White rabbits exposed to lewisite vapor at a concentration of 0.2 mg/L for 2.5 or 7.5 min, followed by a postexposure observation period of up to 28 days. An increase in total corneal thickness was noted starting at day 1 postexposure and epithelial degradation starting at day 3 postexposure, with maximal effect at day 7 postexposure, followed by recovery at later time points. Lewisite also induced an increase in the number of blood vessels and inflammatory cells and a decrease in keratocytes with maximum effects at day 7 postexposure. A significant increase in epithelial−stromal separation was observed at days 7 and 14 following the 7.5 min exposure. Lewisite also caused an increase in the expression levels of cyclooxygenase-2, IL-8, vascular endothelial growth factor, and matrix metalloproteinase-9 at all the study time points, indicating their involvement in lewisite-induced ocular effects.

More recently, Srivasta et al. (2018) identified a molecular mechanism underlying lewisite-induced acute kidney injury. Following cutaneous exposure to lewisite, renal tubular damage was noted in mice. The effect was characterized by loss of brush border in proximal tubules and tubular cell apoptosis accompanied by increases in serum creatinine, neutrophil gelatinase-associated lipocalin, and kidney injury molecule-1. Production of reactive oxygen species in the kidney was noted and resulted in the activation of autophagic and DNA damage response signaling pathways. Scavenging reactive oxygen species by cutaneous postexposure application of the antioxidant *N*-acetyl-L-cysteine, decreased the lewisite-induced autophagy and DNA damage.

11.5 Toxicity

11.5.1 Sulfur mustard

Anslow et al. (1947) and Graef et al. (1948) provided basic information on the clinical pathology and complex

toxicology of sulfur mustards and nitrogen mustards. Subsequently, sulfur mustard toxicity in humans and animals has been extensively reviewed by Sidell et al. (1992), Somani (1992), Watson and Griffin (1992), IOM (1993), ATSDR (2003), NRC (2003), Romano et al. (2008), and Ghanei and Harandi (2011). Sulfur mustard affects the skin, respiratory tract, and eyes. The acute effects include edema, ulceration, and necrosis of epithelial tissue. Systemic toxicity may also occur and is characterized by nausea and vomiting, fever, and malaise. There is evidence of systemic toxicity (gastrointestinal tract) following dermal exposure only (Dacre and Goldman, 1996). Delayed effects include conjunctivitis and blindness following ocular exposure and chronic bronchitis following inhalation exposure. Affected tissues may have an increased susceptibility to secondary infections, and possibility of carcinogenicity of the skin and respiratory tract. However, the often-observed latency between acute exposure and late-onset tissue damage is not fully understood. Of the approximately 100,000 casualties of sulfur mustard exposure during the Iran−Iraq war (1983−88), approximately 45,000 were still experiencing long-term effects of exposure over 20 years later (Ghanei et al., 2010). Similarly, Isono et al. (2018) reported significant long-term autonomic nervous system issues and neuropsychological effects among 44 individuals accidentally exposed to a mixture of sulfur mustard and lewisite in Qiqihar, northeast China, in August 2003. Medical follow-up of the victims was carried out between 2006 and 2014. Various medical issues were detected in 30%−63% of the subjects.

Ambient temperature and humidity govern the degree of toxicity of sulfur mustard; in hot and humid conditions, lower mustard concentrations are required to produce debilitating effects. The severity of sulfur mustard effects is also greater in areas of the body with greater moisture (e.g., the axilla, groin, and eyes). Information regarding the toxic effects of long-term exposure to low levels of sulfur mustard that are not acutely toxic is limited. Ghanei et al. (2010) have reviewed the acute and long-term cutaneous effects of sulfur mustard.

Available data suggest that the location and severity of damage resulting from exposure to sulfur mustard are concentration-dependent and a function of the highly reactive nature of sulfur mustard (Papirmeister et al., 1991). The eyes are generally considered to be the most sensitive and rapidly responding target (Reed, 1918; Reed et al., 1918; Anderson, 1942). For low exposures, sulfur mustard-induced injury appears to be limited to the upper respiratory tract (Eisenmenger et al., 1991) and eyes (Reed, 1918; Reed et al., 1918; Guild et al., 1941; Anderson, 1942). In work with informed volunteer subjects, Anderson (1942) reported that Ct values of 60−75 mg-min/m^3 would result in conjunctivitis, photophobia, and ocular irritation, while Ct values of 75−90 mg-min/m^3 would cause a high proportion of casualties, as determined by more severe ocular damage requiring several weeks of treatment. A chronic ocular injury known as *mustard gas keratopathy (MGK)* has been identified in some individuals following moderate- to high-level exposure. The corneal damage may be recurrent many years following initial exposure. A weak positive correlation between severity of ocular and pulmonary involvement was found in a study of 292 Iran−Iraq conflict veterans (Ghasemi et al., 2012). McNutt et al. (2013) provided insight into the progression and pathophysiological correlates for this injury.

At exposure concentrations higher than those causing ocular damage, pulmonary effects would be expected (Eisenmenger et al., 1991). Regardless of the target tissue, there is a latency period between initial exposure and development of effects. The eyes and respiratory tract appear to have the shortest latency period, with effects appearing within hours depending on the exposure level. Later-onset lung injury is often referred to as mustard lung (ML) because the injury differs somewhat from more typical chronic obstructive pulmonary disease. In a review of the mechanisms of sulfur mustard-induced lung injury, Ghanei and Harandi (2011) found that oxidative stress and apoptosis may be more relevant mechanisms regarding ML than for other chronic obstructive pulmonary diseases.

In addition to the acute toxic effects on the eyes, skin, and respiratory tract, both acute and longer-term neuropsychiatric effects [e.g., depression, anxiety, neurasthenia, insomnia, and posttraumatic stress syndrome (PTSD)] have been documented for individuals exposed to sulfur mustard (Romano et al., 2008). Many of these effects have been documented for individuals exposed during noncombat conditions (e.g., those of munitions plant workers) and are not always the result of high-level exposure that result in serious overt effects. Longer-term effects, such as chronic bronchitis, have been associated with occupational exposures that included episodes of acute toxicity, and delayed or recurrent keratitis may occur 8−40 years after a severe vapor exposure. Sulfur mustard-induced immunosuppression resulting in greater susceptibility to infections has also been reported.

Results of a recent study by Dachir et al. (2017) showed that a 10-minute whole-body exposure of male rats to sulfur mustard at concentrations of 135, 145, or 155 μg/L produced effects typical of sulfur mustard exposure. These included ocular and upper respiratory tract damage within 24−48 h. Examinations of animals euthanized at various times up to 14 days postexposure revealed a progression of ocular and respiratory tract effects as well as alopecia, transient body weight, and aggressive behavior. The remaining rats were euthanized at 12 weeks after exposure. At 4 weeks, clinical signs had resolved. Histopathology of the respiratory tract showed

focal areas of damage and continuation of inflammatory processes. Ocular damage (corneal erosion, abnormal epithelia, and severe edema) continued and remained at study termination.

Additional acute lethality data in animals are summarized in Table 11.7. Based upon the animal data, interspecies variability in the lethal response to sulfur mustard vapor is less than an order of magnitude. For nonlethal effects, the animal data suggest that test species exhibit signs of toxicity that are qualitatively similar to humans when acutely exposed to sulfur mustard vapor. Ocular and respiratory tract irritations are clearly evident in studies using dogs, rats, mice, rabbits, and guinea pigs.

Effects of orally administered sulfur mustard in rats were studied by Sasser et al. (1996a). Repeated gavage administration of sulfur mustard in sesame oil produced epithelial hyperplasia of the forestomach at the highest dose tested, but no deaths and no other treatment-related pathological lesions or changes in clinical chemistry or hematological parameters.

Results of a multigenerational study in rats given sulfur mustard by gavage showed no significant adverse effects on reproductive parameters at any dose level, but revealed dose-related lesions of the squamous epithelium of the forestomach (acanthosis and hyperplasia) (Sasser et al., 1996b). It is likely that the forestomach lesions were a function of the treatment regimen, whereby the bolus dose in an oil vehicle (sesame seed oil) would enhance the effects of sulfur mustard having direct contact with the forestomach tissue. Studies by Hackett et al. (1987) in which rabbits were gavage-dosed with sulfur mustard were equivocal regarding reproductive/developmental effects, due in part to the dose regimen and overt maternal toxicity.

Developmental and reproductive effects of sulfur mustard have been reviewed by the National Research Council (NRC, 1999, 2003). Acute exposures resulting in systemic uptake may have effects on reproductive organs, including inhibition of spermatogenesis. Fetal anomalies were observed in tests with rats given sulfur mustard during gestation, but only at maternally toxic doses.

TABLE 11.7 Acute lethality of sulfur mustard in laboratory species following inhalation exposure.

Species	Lethality value	Concentration (mg/m^3) and exposure duration (min)	References
Rat	2 min LCt_{50}: 1512 mg-min/m^3	756 mg/m^3 (2 min)	Fuhr and Krakow (1945) (not verified)
	30 min LCt_{50}: 990 mg-min/m^3	33 mg/m^3 (30 min)	
	60 min LCt_{50}: 840 mg-min/m^3	14 mg/m^3 (60 min)	
Mouse	2 min LCt_{50}: 4140 mg-min/m^3	2070 mg/m^3 (2 min)	Fuhr and Krakow (1945) (not verified)
	30 min LCt_{50}: 1320 mg-min/m^3	44 mg/m^3 (30 min)	
	60 min LCt50: 860 mg-min/m^3	14.3 mg/m^3 (60 min)	
	60 min LCt_{50}: 42.5 mg/m^3	42.5 mg/m^3 (60 min)	Vijayaraghavan (1997)
Monkey	10 min LCt_{50}: 800 mg-min/m^3	80 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Dog	10 min LCt_{50}: 600 mg-min/m^3	60 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Cat	10 min LCt_{50}: 700 mg-min/m^3	70 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Goat	10 min LCt_{50}: 1900 mg-min/m	190 mg/m^3 (10 min)	Rosenblatt et al. (1975)
Guinea pig	5 min LCt_{50}: 800 mg-min/m^3	160 mg/m^3 (5 min)	Langenberg et al. (1998)
	10 min LCt_{50}: 1700 mg-min/m^3	170 mg/m^3 (10 min)	Rosenblatt et al. (1975)

The genotoxicity of sulfur mustard is well documented as demonstrated by DNA cross-linking, mutations following replication or repair errors, chromosomal breaks, and chromosomal aberrations. Occupational exposures have been associated with increased frequencies of somatic cell mutations, sister chromatid exchanges, and chromosome abnormalities. Studies with rats indicate that subchronic inhalation or oral exposures can produce dominant lethal effects.

The carcinogenicity of sulfur mustard in animals has been reviewed in IARC (1975), Watson et al. (1989), IOM (1993), NRC (1999), and USACHPPM (2000). McNamara et al. (1975) studied the potential carcinogenicity of sulfur mustard in rats, mice, rabbits, guinea pigs, and dogs exposed via inhalation for up to 1 year. No tumors were detected in the mice, rabbits, guinea pigs, or dogs, but skin tumors (i.e., basal and squamous cell carcinomas, trichoepitheliomas, and keratoacanthomas) were associated with sulfur mustard exposure at the highest exposure tested (0.1 mg sulfur mustard/m^3 for 6.5 h, followed by 0.0025 mg sulfur mustard/m^3 for 17.5 h/day, 5 days/week). An increased incidence of pulmonary tumors in Strain A mice was observed following intravenous injections (four doses over 2 days) of sulfur mustard (Heston, 1950), and an increase in injection-site tumors in mice given subcutaneous injections of sulfur mustard over a 6-week period (Heston, 1953).

In a cohort study reported by Easton et al. (1988), there was a significant excess of laryngeal, pharyngeal, upper respiratory tract, and lung cancers in workers at a sulfur mustard manufacturing facility during World War II. A study of the Iranian military veterans exposed to sulfur mustard under battlefield conditions during the Iran–Iraq conflict at levels sufficient to cause severe signs of toxicity indicated a potential increased incidence of chronic myelocytic leukemia (CML). In several earlier studies on World War I veterans who were exposed to sulfur mustard, leukemia was not identified as a possible effect, although it is unclear if examination for CML had ever occurred in those populations. Confounders, such as exposure to benzene or radiation, which complicate the analysis, have not yet been ruled out in the ongoing epidemiologic study of Iranian veterans. Two cases of CML were reported for Japanese workers exposed to sulfur mustard (Shakil et al., 1993) but the incidences of CML in the entire population of sulfur mustard-exposed workers and in an unexposed control population were not reported. Studies in animals provide supporting evidence for the carcinogenicity of sulfur mustard, although the results of some studies are compromised by insufficient exposure durations and injuries resulting from caging situations.

11.5.2 Nitrogen mustards

Information regarding the toxicity of nitrogen mustards is less extensive than that for sulfur mustard. Limited lethality data in animals are summarized in Table 11.8. Like sulfur mustard, exposure to nitrogen mustards may cause skin blistering, as well as respiratory tract injury and ocular damage. Response data from tests with informed human volunteer subjects (NDRC, 1944) suggested a

TABLE 11.8 Lethality of nitrogen mustard (HN2).

Route	Species	Dose (mg/kg)	Exposure time	Effect	References
Oral	Rat	10–85	–	LD_{50}	NDRC (1946)
	Mouse	10–20	–	LD_{50}	Fox and Scott (1980)
Percutaneous	Rat	14	–	LD_{50}	NDRC (1946)
		12	96 h	LD_{50}	Vojvodić et al. (1985)
	Mouse	29–35	–	LD_{50}	NDRC (1946)
	Monkey	<50	–	LD_{50}	NDRC (1946)
Subcutaneous	Rat	6	–	LD_{50}	Vojvodić et al. (1985)
	Mouse	1.4	–	LD_{50}	Fox and Scott (1980)
		2.6–4.5	–	LD_{50}	NDRC (1946)
Intraperitoneal	Rat	1.8–2.5	–	LD_{50}	Fox and Scott (1980)
	Mouse	4.4	–	LD_{50}	Fox and Scott (1980)
Intravenous	Rat	1.1	–	LD_{50}	NDRC (1946), Fox and Scott (1980)
	Mouse	~2	–	LD_{50}	NDRC (1946)

TABLE 11.9 Estimated effect thresholds in humans exposed to nitrogen mustard vapors.

HN1	HN2	HN3	Effect
–	0.012 mg-min/m^3	–	No observable effect level during therapeutic use of HN2 (Van Vloten et al., 1993)
90 mg-min/m^3	70 mg-min/m^3	42 mg-min/m^3	Moderate but reversible ocular effects (Porton Report, 1942a,b, 1943a,b,c,d; U.S. Army Med. Div., 1945a,b; NDRC, 1946)
>21,170 mg-min/m^3	5800 mg-min/m^3	1800 mg-min/m^3	Median blistering Ct (10 or 20 min exposure) for normal skin
		31,300 mg-min/m^3	Median blistering Ct (20 min exposure) for sweating skin (NDRC, 1944)

relative potency of HN3 > HN1 > HN2 for vesicant effects, although the differences were minor. Like sulfur mustard, dermal effects were enhanced by moisture (as from sweating). Estimated thresholds for skin blistering and ocular injury are summarized in Table 11.9. Ocular injury (irritation resulting in compromised operational effectiveness of military personnel) was detected at exposures much lower than those causing dermal effects. All of the toxic effects of nitrogen mustard appear to involve a latency period of several hours for ocular responses and several days for dermal blistering. Nitrogen mustards are alkylating agents with known mutagenicity, but there are no animal cancer bioassays and no human carcinogenicity data.

Nitrogen mustard and its hydrochloride salt have been shown to be teratogenic in mice and rats. Intraperitoneal administration of HN2-hydrochloride/g to mice during gestation resulted in serious teratogenic effects (Danforth and Center, 1954). Haskin (1948) and Murphy et al. (1958) reported similar findings in rats given HN2-hydrochloride/kg subcutaneously during gestation.

Nitrogen mustards are bifunctional alkylating agents that produce a carcinogenic response (primarily lung tumors and lymphomas) in mice following subcutaneous, intraperitoneal, and intravenous administration, as well as by skin painting (IARC, 1987). Intravenous administration of nitrogen mustard to rats produced tumors in multiple organs (IARC, 1987). Information in humans is limited to reports of squamous cell carcinomas of the skin following therapeutic application of nitrogen mustard in the treatment of mycosis, fungoides, and psoriasis (IARC, 1987).

11.5.3 Lewisite

The toxicology of lewisite has been reviewed by Goldman and Dacre (1989), Trammell (1992), Watson and Griffin (1992), Hurst et al. (2008), and NRC (2013). Because lewisite L-2 and L-3 are of lower volatility than L-1, occur in lesser amounts, and are toxicologically similar to L-1, the discussion of toxicity is limited to lewisite (NRC, 2013). Its characteristic geranium-like odor is detectable at 14–23 mg/m^3 (Gates et al., 1946). Lewisite may be lethal to humans following inhalation, dermal, or oral exposure. It is reportedly immediately highly irritating at estimated concentrations of 6–8 mg/m^3. Gates et al. (1946) estimated an LC_{50} of 3300 mg/m^3 for 30 min for lewisite vapor absorption through the bare skin and an inhalation LC_{50} of 120 mg/m^3 for 10 min and 50 mg/m^3 for 30 min. Inhalation of 10 mg/m^3 lewisite for 30 min may result in severe intoxication and incapacitation lasting for several weeks, and inhalation of 10 mg/m^3 for 15 min caused inflammation of the eyes and swelling of the eyelids (Franke, 1977). Like sulfur mustard, moist tissues are particularly sensitive to lewisite. The eyes exhibit the greatest sensitivity (IOM, 1993).

The vesicant properties of lewisite result from direct contact with the skin. Signs of dermal toxicity (pain, inflammation) may be experienced within a minute after exposure. Acute lethality is usually the result of pulmonary injury. Ocular exposure may result in corneal necrosis. Due to its lipophilicity, percutaneous absorption of lewisite is rapid and, at a sufficient exposure, may be associated with systemic toxicity characterized by pulmonary edema, diarrhea, agitation, weakness, hypothermia, and hypotension (IOM, 1993). The threshold for severe systemic toxicity in humans following dermal exposure to lewisite has been estimated at 10 mg/kg (9.1–13.4 mg/kg) (Sollman, 1957).

Eldridge (1923) conducted tests on human volunteers to assess the effects of dermal exposure to lewisite vapor. The arms of men (1–7 men with previously determined average sensitivity to lewisite) were exposed to varying concentrations of lewisite vapor for periods ranging from 10 min to 3 h for the purpose of determining the concentration of lewisite required to create blistering. The resulting dermal responses ranged from reddish discoloration to a clear, watery blister over the entire burned area, accompanied by reddening, swelling, and hardening of the surrounding skin. The burns reached maximum severity in

36–48 h, and healing was complete in 6 days to 2 weeks. The men also reported that the healed skin remained sensitive for several weeks after the healing was complete.

It has been hypothesized that fatalities following dermal exposure to lewisite may be due to blood plasma loss resulting from extensive capillary damage (lewisite shock; Cameron et al., 1946). Sollman (1957) estimated that an oral dose of as little as 2 mL in an adult human (equivalent to 37.6 mg/kg) may be fatal within several hours. The target tissues and organs for systemic toxicity of lewisite include the liver, gallbladder, urinary bladder, lungs, and kidneys (Cameron et al., 1946; Snider et al., 1990). Generally, there is a data deficiency regarding definitive exposure–response data for lewisite.

In studies with rats, Silver and McGrath (1943) found little difference in the acute lethality of *cis*- or *trans*-lewisite exposed for 10 min. Ten-minute mouse LC_{50} values for the *cis*- and *trans*-isomers were 190 and 200 mg/m^3, respectively. All mice exposed to 240 mg/m^3 lewisite for 10 min died. Clinical signs in dogs exposed for 7.5 or 15 min included immediate continual eye blinking, followed by excessive nasal secretion, lacrimation, and sneezing (Armstrong, 1923). Ocular inflammation and vomiting also occurred in some dogs by the end of the 7.5- and 15-min exposures. Dogs exposed for 30 min or longer exhibited frequent retching, vomiting, extreme salivation, labored breathing, and inflammation of the entire respiratory tract. Necropsy revealed a thick membrane in the nostrils, larynx, and trachea, which was accompanied by purulent bronchitis, hemorrhage, pneumonia, edema, and congestion of the lungs.

Similar to the work on sulfur mustard, Sasser et al. (1989a) conducted experiments in rats given lewisite by gastric intubation (in sesame oil) at doses of 0.01, 0.1, 0.5, 1.0, or 2.0 mg/kg, 5 days/week for 13 weeks. A dose-related response was observed for lethality (deaths in the three highest dose groups) and frequency and severity of forestomach lesions. The forestomach lesion incidence and severity were due, at least in part, to the bolus of dosing and the sesame oil vehicle.

Multigeneration reproductive studies in rats (Sasser et al., 1989b) and teratology studies in rats and rabbits (Hackett et al., 1987) given lewisite by gastric intubation were negative or compromised by concurrent maternal toxicity.

The carcinogenic potential of lewisite is not well understood. In a long-term follow-up study, Krause and Grussendorf (1978) reported the formation of a malignant lesion at the site of contact 8 years following a single, acute dermal exposure of a German soldier accidentally exposed to liquid lewisite on his lower right leg in 1940. The lesion was diagnosed as malignant 8 years later, and 38 years after exposure, the area of contact remained ulcerated and diagnosed as Bowen's disease (an intradermal squamous cell carcinoma). Bowen's disease was also diagnosed in workers at a Japanese lewisite production facility (Inada et al., 1978). Findings in these workers were not conclusive due to concurrent exposure to diphenylcyanoarsine and sulfur mustard. Furthermore, no quantitative estimates of dose or exposure rates were available (Inada et al., 1978).

Increased incidences of cancer mortality (respiratory tract: 14%; digestive tract: 9.6%) in workers from the Okuno-Jima poison gas factory were reported by Wada et al. (1968). When cancer rates were correlated with job classifications, the frequency of respiratory and gastrointestinal tract neoplasms was highest in workers who were involved in the production of sulfur mustard or lewisite, followed by those who worked indirectly with sulfur mustard or lewisite. The lowest frequency occurred in the group having no direct contact with the vesicant agents (Yamakido et al., 1985). Similar to the Bowen's disease findings, the cancer incidences were confounded by the fact that workers were also exposed to sulfur mustard, hydrocyanic acid, diphenylcyanoarsine, chloroacetophenone, and phosgene.

11.6 Risk assessment

Although stockpiles of sulfur mustard are being destroyed, the possibility of its use by militant states and terrorists remains. Encountering blister agents in buried munitions has also occurred and the potential for such encounters remains, as do accidents in handling contaminated material. These activities necessitate the need for health-based exposure values for use in emergency planning and emergency response activities.

11.6.1 Sulfur mustards

11.6.1.1 Noncancer

Various standards and guidelines have been developed for sulfur mustard. These values are applicable to occupational exposures, emergency planning and response efforts, and remediation efforts. Airborne exposure limits (AELs) and health-based environmental screening levels (HBESLs) for sulfur mustard have been developed by the US Army (USACHPPM, 1996, 2000). Most health-based criteria for sulfur mustard vapor exposure are based upon protection of the eyes and respiratory tract, which are the most sensitive targets.

Acute exposure guideline levels (AEGLs) for sulfur mustard have been developed for emergency planning and emergency response applications (NRC, 2003; US EPA, 2012). The AEGLs represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h (Table 11.10). The AEGLs for nitrogen mustards are interim status

TABLE 11.10 Inhalation standards and guidelines for sulfur mustard.

Guideline	Exposure duration				
	10 min	30 min	1 h	4 h	8 h
AEGL-1[a]	0.06 ppm	0.02 ppm	0.01 ppm	0.003 ppm	0.001 ppm
AEGL-2[a]	0.09 ppm	0.03 ppm	0.02 ppm	0.004 ppm	0.002 ppm
AEGL-3[a]	0.59 ppm	0.41 ppm	0.32 ppm	0.08 ppm	0.04 ppm
Dept. of the Army/Civilian Occupational WPL[b]					0.0005 ppm (24 h)
Dept. of the Army/Civilian GPL[c]					$1.5 \times 10 \times 10^{-5}$ ppm (24 h)
CDC-CSEPP[d]					0.3 ppm

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. The AEGL-1 values for sulfur mustard are based upon minor ocular irritation in informed human volunteers (Anderson, 1942). AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. The AEGL-2 values for sulfur mustard are based on marked conjunctivitis, edema, photophobia, and eye irritation in informed human volunteers (Anderson, 1942). AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 values for sulfur mustard are based on a lethality threshold estimated in mice (Kumar and Vijayaraghavan, 1998).

[a]*AEGL (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels—AEGL-1, AEGL-2, and AEGL-3—are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects.*

[b]*Worker population exposure limit (DA, 1991, 1997; DHHS, 1988), 8-h TWA, 5 days/week.*

[c]*General population limit (no observable effects), 24-h TWA, 5 days/week.*

[d]*Recommended acute effect levels for determining emergency evacuation distances in the Chemical Stockpile Emergency Preparedness Program (CSEPP); no specified exposure duration.*

values that have not undergone final review and publication by the National Research Council/National Academy of Sciences. Provisional Advisory Levels (PALs) are developed by the US Environmental Protection Agency (EPA) as public emergency exposure limits for three severity levels (PAL 1, PAL 2, and PAL 3) and for exposure durations of 24 h, 30 days, 90 days, and 2 years for both oral and inhalation exposure (Young et al., 2009). Oral PALs for sulfur mustard are not recommended due to insufficient data and because of the chemical nature of sulfur mustard (Bast et al., 2013). Inhalation PAL 1 values for sulfur mustard are 0.00083 mg/m^3 for 24 h and 0.00010 mg/m^3 for 30 days, 90 days, and 2 years. Inhalation PAL 2 values are 0.0042 mg/m^3 for 24 h, 0.0029 mg/m^3 for 30 days, and 0.00097 mg/m^3 for 90 days and 2 years. PAL 3 values are 0.088 mg/m^3 for 24 h, but are not recommended for longer durations (Bast et al., 2013).

An interim reference dose (RfDi), an estimate of a daily dose to humans that is likely to be without appreciable risk of deleterious health effects during a lifetime, of 7×10^{-2} mg/kg/day has been developed for sulfur mustard (NRC, 1999).

Inhalation standards and guidelines for sulfur mustard are summarized in Table 11.10. Other standards and guidelines for sulfur mustard have been summarized by ATSDR (2003).

11.6.1.2 Cancer

The International Agency for Research on Cancer (IARC) classified sulfur mustard as a Group 1 carcinogen (carcinogenic to humans) (IARC, 1987). The National Toxicology Program (NTP) considers mustard gas to be a substance "known to be a human carcinogen" (DHHS, 1988). These assessments are based upon human and animal data.

Studies of occupational exposures to sulfur mustard indicate an elevated risk of respiratory tract and skin tumors following long-term exposure to acutely toxic concentrations. Overall, several factors are important regarding the assessment of the carcinogenicity of sulfur mustard. Increased cancer incidence in humans appears to be associated only with exposures that caused severe acute effects, and occupational exposures tended to involve repeated exposures and repeated injury of the same tissues. Although numerous individuals have suffered sulfur mustard-induced injuries in various conflicts over the years, incidences of skin cancer are rarely reported (Firooz et al., 2011). Evidence for lung cancers resulting from sulfur mustard exposure are well documented, with most cases associated with long-term exposures, although there is emerging evidence for the involvement of acute and short-term exposure (Ghanei and Harandi, 2010). Because the therapeutic use of the sulfur mustard analog nitrogen mustard is associated with an increased incidence of CML,

the reports of CML in HD-exposed individuals appear to be relevant to the carcinogenicity of sulfur mustard.

Cancer slope factors and unit risk values for sulfur mustard have been summarized by ATSDR (2003).

11.6.2 Nitrogen mustards

11.6.2.1 Noncancer

Very few standards and guidelines are available for nitrogen mustards. AEGL values for the nitrogen mustards (HN1, HN2, and HN3) have been developed and are based upon ocular irritation in human volunteers (AEGL-2) and lethality in rodents (AEGL-3), as shown in Table 11.11. Data were insufficient for derivation of level AEGL-1 values. The AEGL values are currently awaiting finalization. The US Army (USACHPPM, 1996, 2004) has developed worker population limit (WPL) values and general population limit (GPL) values for nitrogen mustard (USACHPPM, 1996, 2004).

11.6.2.2 Cancer

Data are not available with which to quantitatively assess the cancer risk from nitrogen mustards. However, IARC (1987) considers nitrogen mustard a Group 2A carcinogen based upon limited evidence in humans and sufficient evidence in animals.

11.6.3 Lewisite

11.6.3.1 Noncancer

The US Army has developed HBESLs for lewisite (USACHPPM, 1999). Additionally, a chronic oral reference dose (RfD) of 1×10^{-4} mg/kg/day is available (NRC, 1999; USACHPPM, 1999), as are inhalation and dermal RfDs (USACHPPM, 1999). Acute exposure guideline levels (AEGLs) have been developed for lewisite (NRC, 2013), and are presented in Table 11.12. Inhalation PAL 1 values are not recommended for lewisite. Inhalation PAL 2 and 3 values for 24-h exposure durations are 0.01 and 0.037 mg/m^3, respectively (Bast et al., 2013). Exposure values for longer PAL-specific durations (30 days, 90 days, and 2 years) are not recommended.

11.6.3.2 Cancer

Data regarding the potential carcinogenicity of lewisite are anecdotal and insufficient for a quantitative assessment. Although quantitative data are lacking, the position maintained by the Centers for Disease Control and Prevention (CDC) (DHHS, 1988) is that some evidence suggests that lewisite may be a carcinogen. For environmental exposure and remediation concerns, the arsenic component and/or arsenic-containing degradation products, however, are relevant.

Although the carcinogenicity of lewisite is equivocal and a quantitative assessment is not feasible, several lewisite degradation products are known carcinogens. Combustion products of lewisite include the inorganic arsenicals, arsenic trichloride, arsenic trioxide, and vinyl chloride. Inorganic arsenic is carcinogenic in humans and animals and is classified as a Group A carcinogen by the US EPA (2008). Arsenic trioxide and vinyl chloride are both considered Group A carcinogens by the US EPA (1984) and Group 1 carcinogens by IARC (1987).

11.7 Treatment

11.7.1 Sulfur mustards

Medical management of sulfur mustard exposure begins with preventing exposure. As previously noted in this chapter, the military use of sulfur mustard necessitated

TABLE 11.11 AEGLs for nitrogen mustards (HN-1, HN-2, HN-3).

Guideline	Exposure duration				
	10 min	30 min	1 h	4 h	8 h
AEGL-1[a]	NR	NR	NR	NR	NR
AEGL-2[a]	0.13 mg/m^3	0.044 mg/m^3	0.022 mg/m^3	0.0056 mg/m^3	0.0028 mg/m^3
AEGL-3[a]	2.2 mg/m^3	0.74 mg/m^3	0.37 mg/m^3	0.093 mg/m^3	0.047 mg/m^3

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. No AEGL-1 values for nitrogen mustards are currently recommended due to insufficient data. AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. A threshold for ocular irritation in informed human volunteers is the basis for the AEGL-2 values for nitrogen mustards. AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 values for nitrogen mustards are based on an estimated lethality threshold in rats.

[a]*AEGL (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels—AEGL-1, AEGL-2, and AEGL-3—are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects.*

TABLE 11.12 AEGLs for lewisite.

Guideline	Exposure duration				
	10 min	30 min	1 h	4 h	8 h
AEGL-1[a]	NR	NR	NR	NR	NR
AEGL-2[a]	1.3 mg/m^3	0.47 mg/m^3	0.25 mg/m^3	0.070 mg/m^3	0.037 mg/m^3
AEGL-3[a]	3.9 mg/m^3	1.4 mg/m^3	0.74 mg/m^3	0.21 mg/m^3	0.11 mg/m^3

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. No AEGL-1 values for lewisite are currently recommended due to insufficient data. AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. A threefold reduction of the AEGL-3 values for lewisite were considered an appropriate and defensible estimate for the AEGL-2 values (NRC, 2013). AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 values for lewisite were based upon an estimated LC_{01} in dogs (Armstrong, 1923).

[a]*AEGL (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels—AEGL-1, AEGL-2, and AEGL-3—are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects.*

full body protection. As a result, considerable effort has been expended in the development and evaluation of protective clothing and equipment (Schier et al., 2005). In general, these include air-purifying and atmosphere-supplying respirators and chemical-protective clothing (e.g., chemical and vapor impermeable coverings and clothing treated with adsorbing or neutralizing chemicals).

Following exposure, rapid decontamination is essential and may include removal of contaminated clothing and removal or neutralization of the agent. Developing medical management strategies for mustard-induced cutaneous damage is an ongoing effort (Graham et al., 2005, 2009; Tewari-Singh and Agarwal, 2016). The use of possible antidotes such as antioxidants has been reviewed by Smith et al. (2008) and Laskin et al. (2010).

Polyurethane sponges containing detoxification additives were developed and evaluated for decontamination and detoxification (Gordon et al., 2006). Using ^{14}C-labeled sulfur mustard and porcine skin, Matar et al. (2019) found that itaconic acid. *N*,*N*'-methylenebisacylamide, 2-trifluoromethylacrylic acid, Fuller's earth, and Fast-Act were effective in reducing the amount of radiolabel absorbed into the skin at 24 h postapplication. A wide range of antiinflammatory drugs and scavengers of reactive oxygen species also continue to be examined.

Ocular exposure requires rapid removal of the agent from the eyes by flushing with water. Symptomatic treatment with analgesics, antiinflammatory agents, and antibiotics is generally appropriate. Jadidi et al. (2014, 2015) reported that topically applied cyclosporin A was useful in treating sulfur mustard-induced superficial eye injuries.

Vapor exposure will also target the respiratory tract and will necessitate respiratory support. Medical management has traditionally relied on prevention, decontamination, and palliative treatment of signs and symptoms. Keyser et al. (2014) reviewed the use of antiinflammatory compounds, antioxidants, protease inhibitors, and antiapoptotic agents. The importance of preventing cell death with the antiapoptotic compounds was emphasized. Veress et al. (2015) found that intratracheal administration of tissue plasminogen activator eliminated mortality and improved morbidity in rats exposed to lethal levels of sulfur mustard vapor. Development of medical countermeasures has been reviewed by Munro et al. (1990), Keyes et al. (2005), Laskin et al. (2010), and Keyser et al. (2014).

11.7.2 Nitrogen mustards

Medical management of nitrogen mustard exposure is similar to that for sulfur mustard. It involves preventing exposure and, where exposure has occurred, decontamination and support therapy. The use of antioxidants in the treatment of nitrogen mustard toxicity is under investigation (Hardej and Billack, 2006).

11.7.3 Lewisite

Similar to the mustard agents, exposure prevention is the first line of defense against lewisite. Rapid decontamination is especially relevant to lewisite exposure due to the rapid development of pain (1−2 min) associated with lewisite exposure. Unlike other vesicants, an effective antidote for lewisite toxicity exists in the form of BAL (2,3-dimercaptopropanol), which binds with arsenicals, thereby countering the lewisite-induced damage. Such chelation therapy is associated with notable side effects (e.g., renal effects) and requires careful medical management. More effective analogs of BAL have been developed that have less significant side effects.

Mouret et al. (2013) reported that for the SKH-1 hairless mouse model, topical applications of dimercapto-chelating agents, such as BAL and meso-2,3-dimercapto-succinic acid (DMSA), were more effective than subcutaneous administration in the attenuation of lewisite vapor-induced injury. Although both agents reduced neutrophil infiltration, wound size, and necrosis of the skin barrier, BAL was found to be more effective than DMSA.

In spite of extensive research efforts, effective therapies for lewisite-induced ocular corneal injuries are not currently available. Goswami et al. (2016) have developed primary human corneal epithelial cell and rabbit corneal organ culture models that may lead to the development of therapies against lewisite-induced ocular effects.

11.8 Concluding remarks and future directions

Vesicants continue to receive attention with respect to the destruction of agent stockpiles, remediation of contaminated sites, the documented and speculated use of these agents in regional conflicts, and their potential use in subversive/terrorist activity. This elevated interest profile has resulted in summaries of older toxicological data, generation of new data, and a greater understanding of the effects of these agents on biological systems. Application of these data is invaluable in the development of various health-based criteria, standards, and guidelines for use in remediation efforts, risk planning, and emergency response.

In accordance with the Chemical Weapons Convention, the United States is destroying (via chemical neutralization followed by biotreatment or supercritical water oxidation) its stockpile of sulfur mustard munitions stored at the Pueblo Army Depot (PEO ACWA, 2019). In the United States, sulfur mustard occurs in artillery projectiles and mortar rounds. As of April 1, 2019, 25% of this stockpile has been destroyed with a completion deadline of December 31, 2023. Continuing research efforts are focusing on additional in-depth understanding of the mechanism of action of these agents through the development of experimental models for vesicant-induced injury, and development of therapeutic measures for the prevention and treatment of vesicant-induced injury.

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Chapter 12

Riot control agents

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12.1 Introduction

Nonlethal agents are a broad class of compounds intended to produce transient incapacitation of an individual or individuals. Incapacitating agents and riot control agents (RCAs) are separate classes of nonlethal agents. Although the two classes share the characteristic to incapacitate, a distinction must be drawn between these two types of agents. RCAs differ from incapacitating agents in several respects. RCAs are potent irritants of peripheral chemosensors. They possess relatively short onset and limited duration of action. These substances induce short-term toxic effects that subside within minutes after termination of the exposure. Modern RCAs have also a very high safety ratio compared with incapacitating agents and first-generation RCAs. Compared with RCAs, incapacitating agents are represented by centrally acting neuropharmacological compounds. Many incapacitating agents were developed during the Cold War and produced either limited lethality and/or prolonged morbidity. Consequently, incapacitating agents have been banned by international treaties recognized by the United States, including the Chemical Weapons Convention (CWC). Specifically, the CWC has placed a ban on the development, production, and possession of any chemical weapon intended to cause death or "temporary incapacitation." The United States considers these broad incapacitating agents as chemical warfare agents (CWAs). However, the United States does not recognize RCAs as CWAs; therefore, US policy considers them to be legal for use by civilian police or the military. The CWC does prohibit their use in times of war.

Although the field of nonlethal agents is diverse and interesting, we limit our discussion to only those agents considered to be RCAs. The goal of RCAs is to temporarily incapacitate through irritating the skin and mucosal membranes of the eyes, airways, and digestive tract. As a result of their short-term toxicity, they are used by military and law enforcement personnel to disperse crowds, clear buildings, and quell riots. Whereas RCAs are often thought of as "tear gas" or pulmonary irritants, they encompass more than this terminology would suggest. They are neither gases nor exclusively pulmonary irritants. Historically, RCAs were categorized as lacrimators, sternutators, and vomiting agents based on their predominant toxicity in the eyes, lungs, or digestive tract. This nomenclature is outdated because modern RCAs affect a wide variety of organ systems. This fact will be clearly evident in the subsequent discussion concerning their mechanism of action and toxicity. Today, RCAs comprise a diverse array of chemical compounds with similar toxic effects because of their introduction to the battlefield in the early part of the 20th century. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Food and Drug Administration.

12.2 History

The Chinese were perhaps the first to use pulmonary irritants with their stink bombs (Smart, 1996). The smoke emanating from them was a primitive sternutator designed to harass the enemy. RCAs were used during the Peloponnesian War in the 5th century BC when the Spartans used smoke from burning coal, sulfur, and pitch to temporarily incapacitate and confuse occupants of Athenian strongholds (Thoman, 2002). During antiquity, the Romans used irritant clouds to drive out their Spanish adversaries from hidden dwellings (Robinson, 1971). Almost all of these examples involved the use of incapacitating agents as an offensive tactical weapon as opposed to controlling crowds for defensive purposes.

The first modern RCA ever deployed was bromoacetone (BA) to control riots in Paris in 1912 (Swearengen, 1966). World War I (WWI) marked the modern age of CWAs (Fig. 12.1). During WWI, both German and

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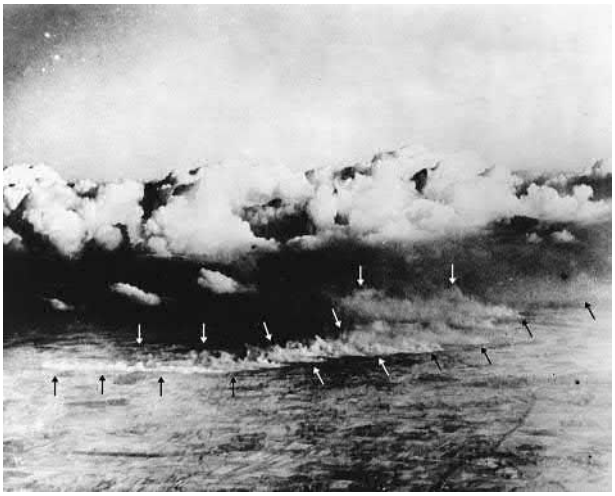


FIGURE 12.1 The birth of CWAs in WWI. The photograph depicts the initial chlorine gas attack by Germany at Ypres, Belgium, on April 22, 1915. The German Army released chlorine gas from cylinders to form a poisonous cloud (indicated by black and white arrows) directed toward the French lines by the prevailing winds.

French forces used a wide variety of irritating agents, such as acrolein (papite), chloropicrin (PS), and diphenylaminearsine (DM; Adamsite); however, BA was the most widely used lacrimator agent at that time. At the end of WWI, the US military investigated the use of chloroacetophenone (CN) as a chemical irritant. First developed by Graebe in 1869 and formulated as Chemical Mace, CN was the most widely used RCA until World War II (Olajos and Stopford, 2004). Two chemists, Corson and Stoughton (1928), synthesized 2-chlorobenzylidene malononitrile (CS); however, it was not adopted by the military as an official RCA until 1959. As a more chemically stable compound and having a greater potency with less toxicity than CN, it gradually replaced CN as the preferred RCA. CS was widely used during the Vietnam War to flush the Viet Cong out of the labyrinth of underground tunnels and bunkers throughout Southeast Asia (Fig. 12.2). In the years following the Vietnam War, other militaries adopted CS. Saddam Hussein's forces used it against Iran during the Iran–Iraq War of the 1980s. Today, CS is commonly used by law enforcement agencies and militaries for riot control training, respirator training in boot camps, temporary incapacitation of an assailant, and civil disturbances. A famous case of RCA use by the US Federal government involved CS dissemination on the Branch Davidian cult members in 1993. Because of its high flammability rating, CS was believed to be a large contributor to the inferno that burned down the Waco, TX, compound and killed its inhabitants. Even before fire broke out and destroyed the compound, CS concentrations supposedly ranged from five to 60 times the amount required to deter individuals (Bryce, 2000).

FIGURE 12.2 (Top) US Army Engineers unpack and test a Mighty-Mite blower in the jungles of Vietnam. The Mighty-Mite aerosolized and disperse smoke, CS powder, or other RCA as a means of tunnel denial. (Bottom) American soldiers ("tunnel rats") wearing M28 protective masks just before entry into underground tunnels previously saturated with CS. *Photograph (Top) Courtesy US Army Engineer School, Fort Belvoir, VA.*

During the 1980s and 1990s, the use of CS gas was rapidly on the decline and slowly being replaced by oleoresin capsicum (OC) spray. OC, an extracted resin from *Capsicum* pepper plants, was first developed in the 1970s as an alternative to CN and CS agents. Commercially available OC sprays used by the public are approximately 1% capsaicin, whereas formulations used by law enforcement agencies can contain up to 15% capsaicin. Most recently, a synthetic form of capsaicin called nonivamide, marketed as Captor, gained popularity as a defensive aerosol in the early 1990s (Olajos and Stopford, 2004).

Under the CWC of 1997, RCAs were banned from use as a method of warfare because in high concentrations RCAs are toxic chemicals with the potential to incapacitate individuals for prolonged periods, produce long-term sequelae, and cause death. The CWC allows RCAs to be used in domestic riot control as well as for enforcement

of domestic law and "extraterritorial law enforcement activities undertaken by military forces" (Rosenberg, 2003). These boundaries and definitions, although vague, were clarified in 2003 by President George W. Bush (Schmitt, 2003). Bush authorized the use of tear gas against Iraqi troops for defensive purposes as allowed in Executive Order 11850 of 1975. Many experts believed this would violate the CWC (which was not signed by Iraq) and give Saddam Hussein the power to use chemical agents against the United States under the authority of the Geneva Protocol (Schmitt, 2003). In the end, the use of RCAs by US military and multinational forces remained limited to rioting prisoners at detention centers during the postinvasion phase (Giovanello, 2012).

Currently, the utilization of RCAs for law enforcement purposes is widespread. In 2018, of the 193 states committed to the CWC, 127 declared holding of CS, 68 of CN, 50 of capsaicinoids, 14 of CR, and 17 of "other types" of RCAs (OPCW, 2019).

12.3 Background

12.3.1 The agents and their physicochemical properties

Unlike the majority of chemical agents that are liquid at room temperature, modern RCAs are crystalline solids with low vapor pressure (see Table 12.1). RCAs are typically administered as fine particles, aerosol sprays, or in solutions; therefore, they are not true gases. The inhalation toxicity of RCAs, as well as CWAs, is often indicated by the expression *Ct*. This term is defined as the product of concentration (*C*) in mg/m^3 multiplied by exposure time (*t*) in minutes ($mg\ min/m^3$). LCt50 and ICt50 are conventional terms used to describe airborne dosages that are lethal (L) or incapacitating (I) to 50% of the exposed population. The intolerable concentration (mg/m^3), ICt50, and minimal lethal concentration (mg/m^3) of the most common RCAs are provided in Table 12.1. The ocular irritancy threshold (minimal irritant or minimal effective dose), estimated human LCt50, and safety ratio are provided in Table 12.2 for these same RCAs. The modern RCAs are characterized by a high LCt50, low effective Ct50, low ICt50, low minimal irritating concentration, and large safety index ratio (LCt50/irritancy threshold). As a rule of thumb, clinical signs and symptoms from RCA exposure generally subside within 30 min but may persist, depending on the dose and duration of exposure (Blain, 2003). CN and CS are the classic representative agents of this class of compounds. The toxicity of both is discussed in depth because of the vast volume of literature available for these compounds.

12.3.1.1 Chloroacetophenone

CN is a crystalline solid with a strong, pungent odor (Fig. 12.3). It is dispersed as a smoke, powder, or liquid formulation from grenades or other devices. It is perhaps better known under the trade name Chemical Mace and was once used widely for self-protection. It was also the standard tear gas used by the military (Fig. 12.4) and police personnel. It has been replaced in favor of the less toxic CS for riot control and capsaicin pepper spray for self-defense.

CN exhibits the greatest toxicity among RCAs in use today. Consequently, it has been replaced by compounds with higher safety ratios. CN is 3- to 10-fold more toxic than CS in rats, rabbits, guinea pigs, and mice (Ballantyne and Swanston, 1978). Pathological findings in the lungs tend to be more severe and CN causes far greater edema. CN typically causes an acute, patchy, inflammatory cell infiltration of the trachea, bronchi, and bronchioles, in addition to early bronchopneumonia. CN not only demonstrates greater irritation to the skin than CS but also is a more potent skin sensitizer (Chung and Giles, 1972). Patients frequently exposed to CN are at high risk for development of allergic dermatitis (Penneys, 1971).

12.3.1.2 Ortho-chlorobenzylidene malononitrile

The term CS was adopted after the two chemists, Corson and Stoughton, who synthesized the compound. CS is a white, crystalline powder with a pepper-like odor and low vapor pressure (Fig. 12.5). It exists in three forms: CS, CS1, and CS2. CS identifies the compound in pure form, which is rapidly hydrolyzed after contact with water. CS1 is a micronized powder of crystalline agent containing 5% silica gel characterized by improved dissemination by an explosive burst or dusting apparatus. In CS2 form, the agent is microencapsulated with silicone to increase its weather resistance and flow properties (WHO, 1970).

CS is the most widely used RCA today, although many countries are switching to even less toxic compounds. CS is used by the US Armed Forces for gas discipline training exercises to help new recruits learn the importance of donning their protective masks quickly (Fig. 12.6). It was also used by the United States during the Vietnam War for tunnel denial and crowd control (Fig. 12.7) and by police forces for dispersing violent protests and incapacitating assailants.

12.3.1.3 Dibenz(b,f)-1:4-oxazepine

Dibenz(*b*,*f*)-1:4-oxazepine (CR) (Fig. 12.8) is a potent sensory irritant with less toxicity than CS or CN (Ballantyne, 1977). CR causes an immediate and effective irritation of the eyes, nose, and skin without persistent

TABLE 12.1 Physical characteristics and toxicity data for the common RCAs.

Agent	Discovered in	Physical characteristics			Toxicity data			
		Solubility	Vapor pressure (mmHg at 20°C)	Vapor density	Onset	Intolerable concentration (mg/m^3)	ICt50 (mg min/m^3)	Minimal lethal concentration[a] (mg/m^3)
CS	1928 (Corson and Stoughton)	Insoluble in water Soluble in organic solvents	0.00034	6.5	Immediate	5	3–10	2500
CN	1869 (Graebe)[b]	Poorly soluble in water	0.0054	5.3	Immediate	35	20–40	850–2250
DM	1915 (Wieland)[c] and 1918 (Adams)[d]	Insoluble in water Poorly soluble in organic solvents except acetone	2×10^{-13}	9.6	Delayed with long recovery period	5	22–150	1100–4400
CR	1962 (Higginbottom and Suschitzkey)	Sparingly soluble in water Stable in organic solvents	0.00059	6.7	Immediate	1	1	10,000
Bromobenzyl cyanide (CA)	1881 (Riener)[e]	Insoluble in water Soluble in organic solutions	0.12	4.0	Immediate	0.8	30	1100

[a] *Estimate for minimal lethal concentration (10 min exposure).*
[b] *Graebe (1871).*
[c] *Wieland and Rheinheim (1921).*
[d] *Sartori (1939).*
[e] *Prentiss (1937).*

Source: Based on Maynard, R.L., Ballantyne, B., Marrs, T., Syversen, T., 1999. Toxicology of chemical warfare agents. In: B. Ballantyne, T. Marrs, T. Syversen (Eds.), General and Applied Toxicology. Macmillan Reference, London, England; Olajos, E.J., Salem, H., 2001. Riot control agents: pharmacology, toxicology, biochemistry and chemistry. J. Appl. Toxicol. 21, 355–391; Sidell, F.R., 1997. Riot control agents. In: R. Zajtchuk (Ed.), Medical Aspects of Chemical and Biological Weapons. Borden Institute, Washington, DC; Smith, C.G., Stopford, W., 1999. Health hazards of pepper spray. N. C. Med. J. 60, 268–274.

TABLE 12.2 Health risk considerations for the common RCAs.

Agent	Irritancy threshold[a] (mg/m^3)	Estimated human LCt50[b] ($mg\ min/m^3$)	Safety ratio[c]	Adverse effects
CN	0.3[a]	8500–22,500	28,000	Danger of permanent eye injury, vesiculation, bronchopneumonia, reactive airways, documented fatality cases
CS	0.004[a]	25,000–150,000	60,000	Same as CN, enhanced persistence compared to CN
CR	0.002[a]	>100,000	>100,000	No significant respiratory toxicity, no documented fatality cases
OC	0.0003[d]	Not available	>60,000	Eye, skin, respiratory toxicity, documented fatality cases
DM	~1[a]	11,000–44,000	11,000	No longer used
CA	0.15[a]	11,000	11,000	Predominantly a lachrymatory agent, no longer used

[a]*Ocular irritancy thresholds unless indicated otherwise.*
[b]*Values obtained from references: Maynard et al. (1999); Olajos and Salem (2001); Sidell (1997); Smith and Stopford (1999).*
[c]*Values derived from estimate of the human LCt_{50} (lower bound)/irritancy threshold (minimal effective dose). Therefore, ranges are not provided for the safety ratios.*
[d]*Threshold for respiratory complaints by capsaicinoids (Stopford et al., 2006).*

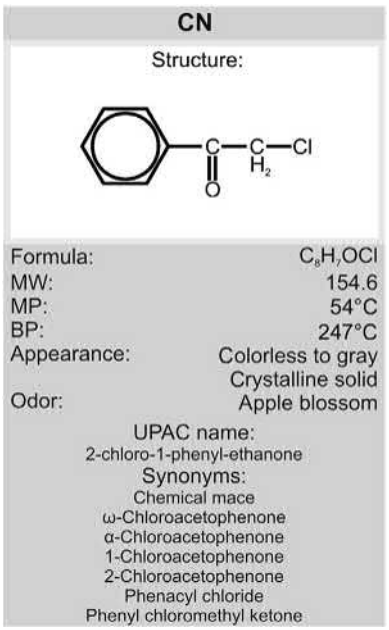


FIGURE 12.3 Chemical structure and physicochemical properties of chloroacetophenone (CN).

effects in these target organs. The irritation associated with CR is more transient compared with other RCAs. It is 5–10 times greater in potency than CS; therefore, a smaller concentration is needed to cause irritation (low minimal irritant concentration or dose) and incapacitation (low ICt50) (Tables 12.1 and 12.2). CR has a favorable safety ratio; it is safer than other RCAs based on its higher LCt50 (Table 12.1) and greater LCt50/irritancy

FIGURE 12.4 US soldier in protective clothing disseminating CN aerosol using the M33A1 disperser.

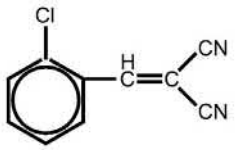
threshold (safety ratio). In humans, the effects caused by CR are identical to CS. The LCt50 for humans is estimated at $>100{,}000\ mg\ min/m^3$. Despite its reduced toxicity in humans, CR is not entirely without risk. CR is fairly stable, resists weathering, and persists in the environment (Sidell, 1997), therefore, enhanced toxicity may occur with prolonged exposure.

12.3.1.4 Diphenylaminechlorarsine

DM (Fig. 12.9) or Adamsite is a pro-emetic agent used in WWI. DM has greater toxicity than other RCAs and has been abandoned in favor of compounds with less toxicity and greater safety ratios. Although toxicity is typically

CS

Structure:



Formula:	$C_{10}H_5N_2Cl$
MW:	188.6
MP:	93°C
BP:	310°C–315°C
Appearance:	White, crystalline solid
Odor:	Pungent, pepper-like

UPAC name:
2-[(2-chlorophenyl) methylidene] propanedinitrile

Synonyms:
2-(2-Chlorobenzylidene) malononitrile
(ortho-Chlorobenzal)malononitrile
2-(Chloro-phenyl)methylenemalononitrile
(2-Chlorophenyl)methylenepropanedinitrile
β,β-Dicyano-ortho-chlorostyrene

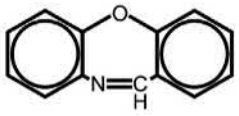
FIGURE 12.5 Chemical structure and physicochemical properties of ortho-chlorobenzylidene malononitrile (CS).

FIGURE 12.6 Aerial spraying of a Chemical Warfare School class with CS tear gas during a training event.

FIGURE 12.7 US Army soldiers using CS tear gas in South Vietnam.

CR

Structure:



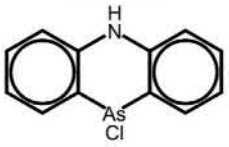
Formula:	C_8H_7OCl
MW:	195.2
MP:	72°C
BP:	335°C
Appearance:	Pale yellow, crystalline solid
Odor:	Pungent, pepper-like

Synonym:
Dibenzoxazepine

FIGURE 12.8 Chemical structure and physicochemical properties of dibenz(*b,f*)-1:4-oxazepine (CR).

DM

Structure:



Formula:	$C_{12}H_9AsClN$
MW:	277.5
MP:	195°C
BP:	405°C–415°C
Appearance:	Yellow-green, crystalline solid
Odor:	No distinct odor, but irritating

Synonyms:
Adamsite
5-Aza-10-arsenaanthracene chloride
10-Chloro-5,10-dihydroarsacridine
10-Chloro-5,10-dihydrophenarsazine
Dibenzo-1-chloro-1,4-arsenine
Diphenylaminochloroarsine
Phenarsazine chloride

FIGURE 12.9 Chemical structure and physicochemical properties of diphenylaminechlorarsine (DM).

delayed with DM exposure, toxic signs and symptoms can occur within minutes after exposure. Systemic toxicity may also be more pronounced and prolonged. Symptoms often subside hours after exposure. Because DM is an antiquated RCA, this compound is irrelevant today and will not be discussed further.

12.3.1.5 Oleoresin capsicum

OC is an oily resin derivative from capsicums and is composed of several related compounds. Capsicums are solanaceous (nightshade species) plants from the genus *Capsicum*. More than 20 species fall within the genus. Capsaicinoids are considered the active ingredients of OC. These active compounds are endocrine products of

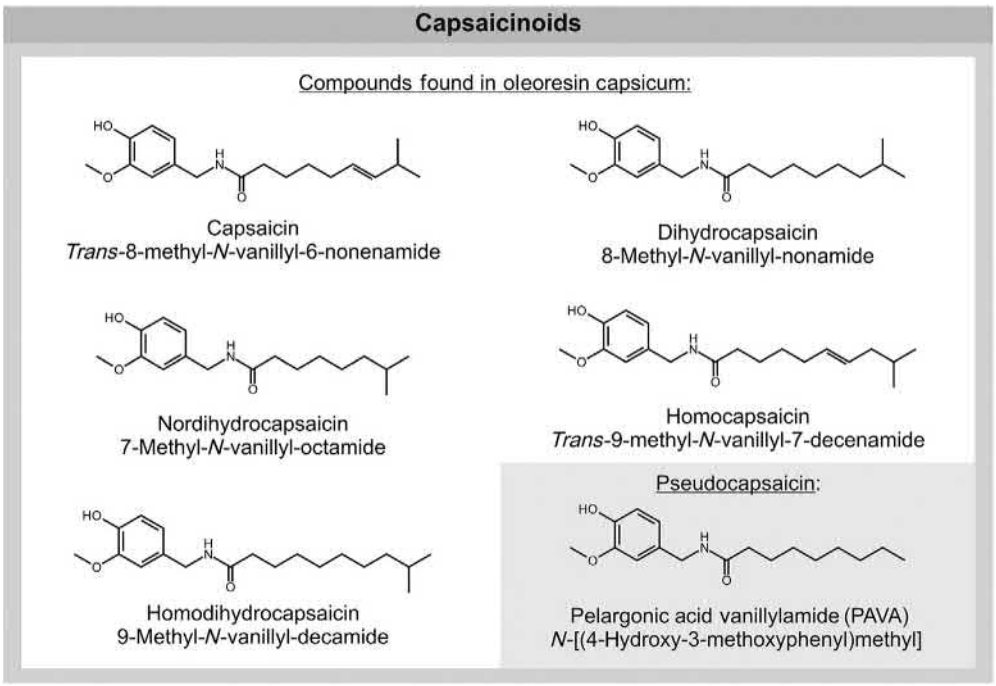


FIGURE 12.10 Chemical structures of the most common capsaicinoids found in OC.

glands found in the plant placenta and are a mixture of two unsaturated and three saturated homologs (Fig. 12.10). Capsaicinoids are isolated through a volatile solvent extraction of the dried, ripened fruit of chili peppers. The capsaicinoids are distilled, dried, and compounded together. The final oleoresin contains several branched-chain alkyl vanillylamides, in addition to capsaicin, the major component in OC. The predominant capsaicinoid components of OC are capsaicin (70%), dihydrocapsaicin (20%), norhydrocapsaicin (7%), homocapsaicin (1%), and homodihydrocapsaicin (1%) (Salem et al., 2006) (Fig. 12.10).

Capsaicinoids cause dermatitis as well as nasal, ocular, pulmonary, and gastrointestinal effects in humans. OC gained popularity in the 1990s as a defensive weapon for civilians and law enforcement agencies because they produce immediate, temporary immobilization and incapacitation when sprayed directly into the face or eyes. It is important to note that hand-held pepper spray formulations can contain OC by themselves or a mixture of OC and CS.

12.3.1.6 Pelargonic acid vanillylamide

Other capsaicinoids are available. Pelargonic acid vanillylamide (PAVA or nonivamide), shown in Fig. 12.10, is a "synthetic" form of capsaicin. Nonivamide was first synthesized by Nelson (1919). Nonivamide was originally found to be a minor component in *Capsicum annum* peppers (Constant et al., 1996); however, the majority of PAVA is derived from synthesis rather than by extraction from natural plant sources. As a result, the composition and concentration of PAVA can remain consistent (Haber et al., 2007).

For PAVA to work, it must be directed at the subject's eyes. The pain in the eyes is reported to be greater than that caused by CS tear gas (ACPO, 2006; Smith et al., 2004). The effects are immediate but will subside 15–20 min after exposure to fresh air. PAVA does display disadvantages. Although PAVA has a high rate of effectiveness, it has proven to be ineffective against those under the influence of alcohol (ACPO, 2006). Additionally, the Smith et al. (2004) study mentions a number of cases in which PAVA was used without effect. The effect of PAVA was also reported to be almost instantaneous, with the undesirable effect that recovery was also immediate. PAVA is commercially available in two forms, captor I and captor II. Captor I contains 0.3% PAVA with a solvent of equal parts of ethanol and water. Captor II contains 0.3% PAVA with propylene glycol, water, and ethanol (COT, 2007).

12.3.1.7 New potent compounds

Identification of CS, CN, and CR as agonists of transient receptor potential ankyrin 1 (TRPA1) implicated in inflammatory pain-related signals in 2008 (Brône et al., 2008) revived scientific interest in RCAs. Since then, new agonists and antagonists have been synthesized. Among them, several morphanthridine and CR derivatives show stronger agonism toward TRPA1 than conventional RCAs

(Gijsen et al., 2010). Their physicochemical properties as well as toxicities are not known. Nonetheless, the substances could provide a higher safety ratio than CR.

12.4 Mechanism of action

The mechanisms of action through which RCAs act are not completely understood. RCAs interact with neurons in afferent nociceptive fibers and target receptors within transient receptor potential (TRP) family of cation-selective channels. Currently, seven different subfamilies can be recognized within this family.

CS, CN, and CR are potent activators of TRPA1, the only member of the ankyrin subfamily found in mammals. These receptors are expressed in neurons and in some non-neuronal cell types, such as keratinocytes, melanocytes, fibroblasts, smooth muscle cells, and glial or endothelial cells (Viana, 2016). TRPA1 is best known as a sensor for environmental irritants, cold, and stretch (Annas et al., 2015). Genetic ablation or pharmacological inhibition of TRPA1 dramatically reduces acute pain behavior in mice after CS, CN, and CR exposure, confirming the essential role of TRPA1 in their sensory detection (Bessac et al., 2009). In human studies, CS, CN, and CR affinity to TRPA1 clearly correlates with their perceived irritancy (Lindsay et al., 2015). CS, CN, and CR are electrophilic alkylating compounds (Brône et al., 2008). From the chemical point of view, the electrophilic activators of the TRPA1 receptor bind to reactive cysteines within the intracytoplasmic N-terminus of the receptor in a covalent fashion by the mechanism known also as the Michael addition (Kádková et al., 2017).

Capsaicinoids selectively interact with transient receptor potential vanilloid 1 (TRPV1) receptor. The receptor is one of six members of the TRPV subfamily named for chemicals with a vanillyl moiety in its structure that activate this channel (Caterina et al., 1997). TRPV1 is coexpressed on the vast majority of TRPA1-expressing sensory nerves as well as on non-neuronal cells. Both receptors integrate a variety of noxious stimuli. TRPV1 is particularly responsive to diverse physical and chemical stimuli, including heat ($>43°C$), low pH (<6.0), or capsaicinoids (Fernandes et al., 2012). TRPV1 knockout mice exposed to capsaicin display significantly reduced pain-related behavioral responses, underscoring the important role of this ion channel (Caterina et al., 2000). Capsaicin interacts with its receptor following the traditional Fischer lock-and-key principle, which was demonstrated using high-resolution cryo-electron microscopy structures of TRPV1 in its apo and capsaicin-bound states (Yang et al., 2018).

Binding of RCAs to TRPA1 or TRPV1 causes channel opening, influx of Ca^{2+}, Na^{+}, and K^{+}, depolarization of the neuron, activation of signaling pathways, alteration of cyclooxygenases, and release of neuropeptides (Liu et al., 2016; Martling, 1987; Yamamoto et al., 2015; Zhang et al., 2007). TRPA1- and TRPV1-expressing sensory neurons release active substances, such as neurokinin A, substance P, and calcitonin gene-related peptide that produce changes in the airway mucosa and induce neurogenic inflammation of the respiratory epithelium, airway blood vessels, glands, and smooth muscle. This is associated with bronchoconstriction, increased vascular permeability, edema of the tracheobronchial mucosa, elevated mucosal secretion, and neutrophil chemotaxis (Tominack and Spyker, 1987). In addition, proinflammatory mediators, including bradykinin, prostaglandins, histamine, purines, and proteases upregulate expression of both channels (Bessac and Jordt, 2010). This positive feedback may explain increased sensitivity of asthmatics to RCA exposure, as well as exacerbation of asthma and inflammatory skin conditions after RCA exposure (Dimitroglou et al., 2015; Watson and Rycroft, 2005).

Additional mechanisms might be involved in toxicity of RCAs. CS, CN, and CR contain chloride atoms in their structure. Production of hydrochloric acid through reduction of chloride ions on mucosal membranes may help explain the marked, focal irritation and burns on the skin resulting from exposure to CS (Anderson et al., 1996; Worthington and Nee, 1999).

CS, CN, and CR are also SN_2 alkylating agents (Ballantyne and Swanston, 1978; Cucinell et al., 1971); in contrast, the vesicant mustard is an SN_1 alkylating agent. The SN_2 moniker describes direct reaction of the agent with nucleophilic compounds in a bimolecular fashion. In particular, they react with intracellular thiol or SH-containing enzymes, thereby inactivating them (Ballantyne et al., 1977). Mackworth (1948) first showed that CN and other first-generation lacrimators used during WWI (bromoacetophenone, ethyl iodoacetate, chloropicrin, and bromobenzyl cyanide) strongly inhibited thiol-containing succinic dehydrogenase and pyruvic oxidase, major players of crucial metabolic pathways. Some suggest that lactic dehydrogenase is completely insensitive to lacrimators (Mackworth, 1948), but only lacrimators from the iodoacetate family were ever studied by this group. Another group reported that lactic dehydrogenase is, in fact, strongly inhibited by CS (Cucinell et al., 1971). CS also reacts with the disulfhydryl form of lipoic acid, a coenzyme in the pyruvate decarboxylase system (Olajos and Salem, 2001). Alteration in dihydrolipoic acid biochemistry can lead to decreased acetyl CoA levels, resulting in cellular injury. Inactivation of these metabolic enzyme systems is, however, transient because the enzymes can be rapidly reactivated if exposure is terminated (Beswick, 1983). Less is known regarding the action of CR on thiol-containing molecules. But according to Brône et al. (2008) both CS and CR rapidly form adducts with benzylmercaptane in vitro.

The metabolism of CS to cyanide (see Section 12.5) was once thought to be responsible for agent-induced lethality in animals (Cucinell et al., 1971; Jones and Israel, 1970). Despite reports of alleged fatality cases, mortality in humans after CS administration has not been authenticated (Ballantyne et al., 1977; Olajos and Salem, 2001). CS has been demonstrated to cause death in dogs (Cucinell et al., 1971). CS is hydrolyzed to malononitrile and 2-chlorobenzaldehyde (Brewster et al., 1987). Further metabolism of malononitrile yields two potential cyanides, which could interact with sulfur thiols to yield thiocyanate. Cyanide typically causes death immediately, but animals administered CS by inhalation far more than the lethal *Ct* do not die immediately; death occurs 12−24 h after exposure. In fact, death seems to be attributable to airway and lung damage (Ballantyne and Swanston, 1978). Studies to ascertain cyanide production after CS exposure in humans showed negligible levels of plasma thiocyanate (Leadbeater, 1973). Another study revealed low levels of cyanide production in mice administered with ^{14}C-labeled CS (Brewster et al., 1987). In short, cyanide is not liberated in sufficient quantities from CS metabolism to become toxic enough to cause death. It may however alter an array of biochemical interactions, including lipid peroxidation (Johnson et al., 1987), neuronal calcium homeostasis (Johnson et al., 1986), and phospholipid hydrolysis (Sakaida et al., 1992), and amplify resulting tissue damage.

Additional mechanisms of action have been also identified in capsaicin-treated models. TRPV1-independent pathways were suggested to block complexes I and III of the respiratory chain, leading to the impairment of mitochondrial potential (Skrzypski et al., 2014). Moreover, capsaicin suppresses the plasma membrane NADH-oxidoreductase electron transport chain (Gonzales et al., 2014; Sánchez et al., 2007). Both mechanisms disrupt cellular redox homeostasis, stimulate ROS overproduction, and contribute to the cytotoxic effect. Interestingly, Thomas et al. (2011) demonstrated in normal and immortalized bronchial epithelial cells that there is a concentration threshold (∼200−250 μM), at which capsaicinoid analogs become cytotoxic independent of TRPV1 expression or function. Capsaicin TRPV1-independent mechanisms were also implicated in the regulation of voltage-gated potassium channels (Yang et al., 2014), lipopolysaccharide-induced fever (Mahmoud et al., 2007), or glycinergic neurotransmission in hypoglossal motor neurons (Thakre and Bellingham, 2019).

12.5 Toxicokinetics

The uptake, distribution, and metabolism of CS, CR, and capsaicins have been well characterized. In contrast, toxicokinetics of CN has been poorly characterized despite numerous investigations reporting its toxicity.

12.5.1 Uptake, distribution, and metabolism of ortho-chlorobenzylidene malononitrile

CS is rapidly absorbed and distributed throughout the body after inhalation exposure. Pharmacokinetic studies show that CS is removed from circulation quickly with first-order kinetics after inhalation exposure. CS half-life is just less than 30 s (Olajos et al., 2004). Short half-lives in the circulatory system are also demonstrated for the major CS metabolites (2-chlorobenzyl malononitrile and 2-chlorobenzaldehyde) (Leadbeater, 1973). The absorption of CS from the digestive tract in cases of exposure by ingestion is unknown at this time. Systemic toxicity has been noted after ingestion of CS pellets (Solomon et al., 2003).

In mammalian species, CS rapidly hydrolyzes to form 2-chlorobenzaldehyde and malononitrile (Leadbeater, 1973). The malononitrile intermediate is further metabolized from two cyanide moieties, which are converted to thiocyanate (Cucinell et al., 1971). The aldehyde intermediate undergoes oxidation to 2-chlorobenzoic acid or reduction to 2-chlorobenzyl alcohol. In rats, these metabolites are conjugated and excreted in the urine (80%−95%) and feces (Brewster et al., 1987). 2-Chlorohippuric acid was demonstrated to be the major CS metabolite. Its urinary concentration correlates with CS concentration during exposure and urine sampling time, showing the potential to be an effective retrospective indicator of CS in future biomarker developments (Buchanan et al., 2017).

12.5.2 Uptake, distribution, and metabolism of dibenz(*b,f*)-1:4-oxazepine

Absorption of CR after aerosol inhalation is rapid, with a plasma half-life of 5 min, this is consistent with half-life estimates after intravenous administration (Upshall et al., 1977) and gastrointestinal uptake (French et al., 1983). Corneal tissue has been demonstrated to take up CR and metabolize it to the lactam derivative (Balfour, 1978).

A number of studies have investigated the bioconversion, fate, and elimination of CR in various animal species (Balfour, 1978; French et al., 1983). Human metabolic studies of CR have not been performed because of the high degree of sensitivity of human tissues to CR. The maximum tolerated dosage is far too low to allow for detection in metabolic studies (Olajos et al., 2004). The lactam derivative dibenz[*b*, *f*]1:4-oxazepin-11-(10H)-one is a primary metabolic product of metabolism and a direct precursor of the urinary hydroxylated metabolites. In rats, the lactam, a dihydro-CR metabolite, an amino alcohol of

CR, and an arene oxide are metabolites in CR degradation. In the rat, the major mechanism for elimination is sulfate conjugation and biliary excretion to a limited extent. Phase I metabolism by microsomal mixed-function oxidases involves reduction of CR to the amino alcohol, oxidation to form the lactam ring, and hydroxylation to form the hydroxylactams. Phase II conjugation reactions sulfate the hydroxylactam intermediates for renal elimination. Amino alcohol intermediates are conjugated with glucuronide for biliary secretion.

12.5.3 Uptake, distribution, and metabolism of capsaicin

Capsaicin is well absorbed when administered topically or orally. Gastrointestinal absorption reaches up to 94%–98% (Leelahuta et al., 1983; Suresh and Srinivasan, 2010). The estimated plasma half-life varies from 0.4 to 1.6 h (Babbar et al., 2009; Chaiyasit et al., 2009). Capsaicin and capsaicinoids undergo phase I metabolic bioconversion to catechol metabolites via hydroxylation of the vanillyl ring moiety (Miller et al., 1983). Metabolism involves oxidative, in addition to nonoxidative, mechanisms. An example of oxidative conversion involves the liver mixed-function oxidase system to convert capsaicin to an electrophilic epoxide, a reactive metabolite (Olajos et al., 2004). Surh and Lee (1995) have also demonstrated the formation of a phenoxy radical and quinine product; the quinine pathway leads to formation of a highly reactive methyl radical (Reilly et al., 2003). Capsaicin dimers are known to be produced by the coupling of phenoxyl and/or carbon-centered radical intermediates (Surh and Lee, 1995). The alkyl side chain of capsaicin also undergoes rapid oxidative deamination or hydroxylation (Reilly et al., 2003) to hydroxycapsaicin as a detoxification pathway. An example of nonoxidative metabolism of capsaicin is hydrolysis of the acid–amide bond to yield vanillylamide and fatty acyl groups. Phase II involves formation of glutathione and *N*-acetylcysteine conjugates that are primarily excreted in the urine (Reilly et al., 2013).

12.6 Toxicity

RCAs produce a wide variety of physiological effects in humans. Fig. 12.11 illustrates toxic signs and symptoms

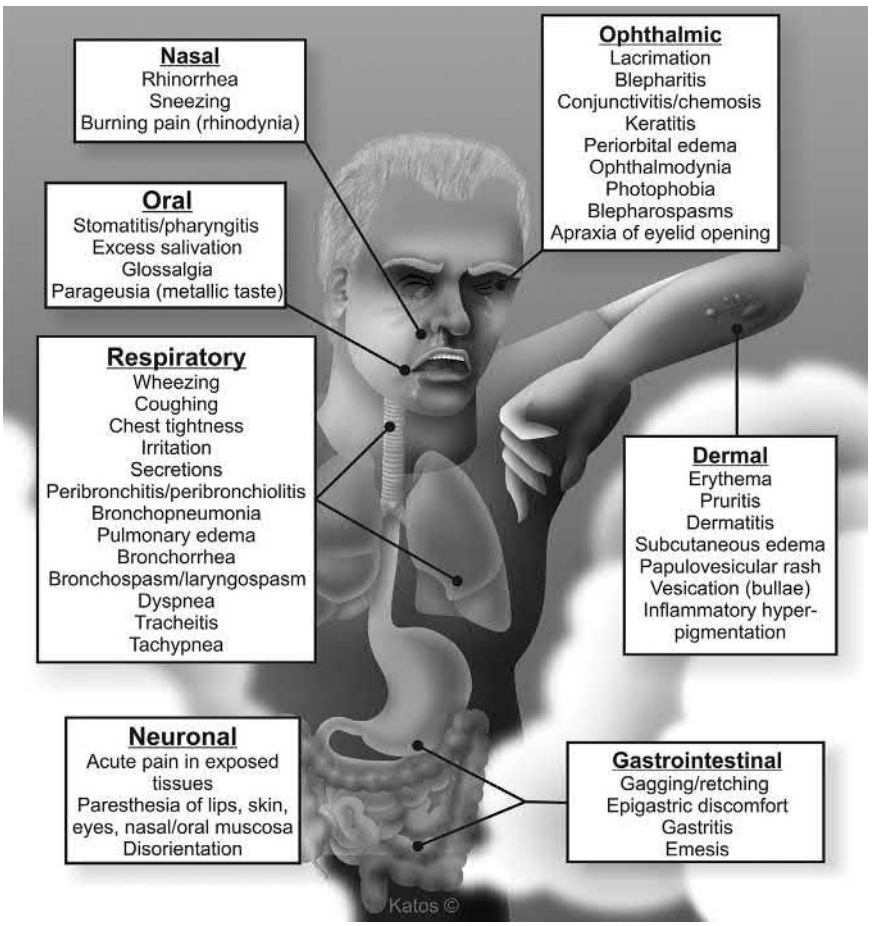


FIGURE 12.11 Physiological effects of riot control agents. *Illustrated, copyright protected, and printed with permission by Alexandre M. Katos.*

of exposure. The clinical effects in the figure are representative of those encountered after CN or CS exposure. CR causes effects qualitatively similar to those caused by CS, except it has greater potency. The predominant anatomical regions affected include eye, lung, and skin. RCAs also cause nasal, oral, neuronal, and gastrointestinal effects.

12.6.1 Ophthalmological effects

The eyes are a major target for the short-lived toxic effects of RCAs. Eye findings from RCA toxicity can range in severity from conjunctival erythema to ocular necrosis. Lacrimation, conjunctival erythema/edema, blepharitis, and erythema are the most typical findings after exposure to all RCAs. Toxic signs may further include periorbital edema (Yih, 1995), blepharospasm or spasms during eyelid closure (Blain, 2003; Grant, 1986), apraxia of eyelid opening, ophthalmodynia, corneal injury, and ocular necrosis (Grant, 1986). Fig. 12.12 illustrates and summarizes the common toxic ophthalmological signs and symptoms associated with RCA aerosol exposure. It is important to note that eye findings tend to be more severe in RCA exposure victims if they are wearing contact lenses (Solomon et al., 2003). The degree of the injury is also higher if eyes were anesthetized prior to the exposure (MacLeod, 1969).

12.6.1.1 Ortho-chlorobenzylidene malononitrile

After exposure to CS, the latency period for the development of ocular signs varies from immediate/few minutes to less than 24 h. Dimitroglou et al. (2015) reviewed 25 studies covering 90 cases of CS exposure. The ocular symptoms were present in 57% of cases and most frequently included lacrimation, eye irritation, conjunctivitis, and stinging of the eyes. There were also reports of

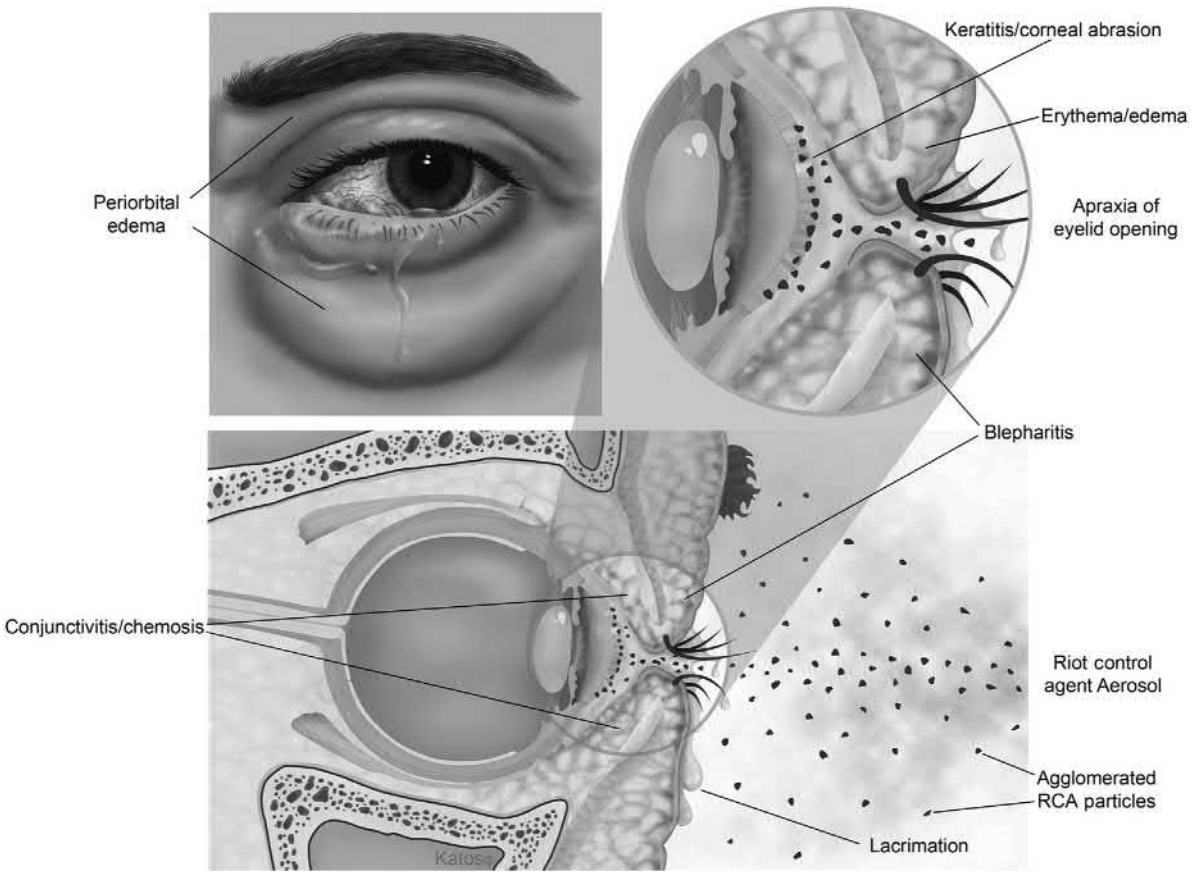


FIGURE 12.12 Exposure of the eye to CS aerosol. (Top left panel) External view of left eye immediately after exposure to CS aerosol, showing scleral injection, periorbital edema, and lacrimation. (Bottom panel) Penetration of CS aerosol into the eye, sagittal view. Following exposure to CS, the eye responds with inflammation, edema (chemosis), lacrimation, erythema, eye pain, and eyelid closure. (Top right panel) Close-up of the eye and eyelids, sagittal view. Inflammation of the eyelids (blepharitis), conjunctivae (conjunctivitis), and cornea (keratitis) are apparent. The eye, in turn, responds with spasms of eyelid closure (blepharospasms) followed by an inability to open the eyelids (apraxia of eyelid opening). Agglomerated CS particles can penetrate the eye on initial contact and cause corneal abrasions. *Illustrated, copyright protected, and printed with permission by Alexandre M. Katos.*

blepharospasm with excessive blinking of the eyes, keratitis, and transient reduction of vision. Recovery is typically complete within 15–30 min after exposure, but a few signs such as erythema of the lid margins and photophobia may persist up to 48 h (Ballantyne et al., 1974), depending on the concentration and duration of exposure (Blain, 2003). The conjunctivae may appear injected or even progress to fulminant conjunctivitis and blurred vision after some RCAs, including CS (Euripidou et al., 2004).

In studies involving human exposure (Rengstorff and Mershon, 1969a,b), CS (0.1% or 0.25% in water; 1.0% in triocyl phosphate) sprayed or administered as ophthalmic drops onto the eyes caused apraxia of eyelid opening with blepharospasm on eyelid closure for 10–135 s. It also caused a transient conjunctivitis but no corneal damage on further inspection with a slit lamp. Rabbit eyes contaminated with CS as a solution (0.5%–10% in polyethylene glycol), as a solid, or thermally dispersed as a smoke (15 min at 6000 mg/m^3) showed greater toxicity with solution. CS in solution caused profuse lacrimation, conjunctivitis, iritis, chemosis, keratitis, and corneal vascularization at concentrations of 1% or more. The lesions tended to be more severe and have a greater duration at higher doses. Histologically, the cornea appeared with patchy denudation of the epithelium and infiltration of neutrophils to the site of injury (Ballantyne et al., 1974).

The eyes are also affected by a CS agent without direct contact between the agent and the eye. In one report, seven patients were exposed to oral ingestion of a juice drink contaminating CS pellets (Solomon et al., 2003). In addition to mild headache and gastrointestinal irritation, they reported ocular irritation and lacrimation. The majority of symptoms resolved within 24 h.

12.6.1.2 Chloroacetophenone

CN causes a similar constellation of ocular signs and symptoms as CS, but CN toxicity is likely to be more severe in the eyes and skin. CN sprayed into the eyes from a distance causes lacrimation, edema of the corneal epithelium and conjunctivae, and reversible epithelial defects of the cornea (Leopold and Lieberman, 1971). Toxic signs in the conjunctivae from CN Mace exposure can include conjunctivitis, sloughing, limbal ischemia, and symblepharon formation (adhesion of the eyelids to the eyeball) (Scott, 1995). Permanent eye injury is unlikely, except after exposure to high concentrations of CN Mace (Grant, 1986). Although permanent eye damage is uncommon, increased intraocular pressure from edema may precipitate acute angle closure glaucoma if left untreated.

Because RCAs are solids, it is possible for a particle to clump or agglomerate, causing penetration into corneal or conjunctival tissues (Fig. 12.12). Agglomerated CN particles can penetrate eye tissue as a result of tear gas cartridge discharge (Levine and Stahl, 1968). CN powder coalesces over time; therefore, the mechanical damage is greater with the use of old cartridges (Laibson and Oconor, 1970). In addition to large powder CN agglomerates, traumatic effects from the propellant charge, wadding, or foreign pieces from the cartridge should also be suspected when evaluating eye damage from CN.

Although RCAs produce short-lived effects, rabbits exposed to 10% CN solution caused iritis and conjunctivitis for more than 7 days and caused corneal opacity (Grant, 1986) lasting longer than 2 months (Gaskins et al., 1972). CS at the same concentration produced moderate conjunctivitis without iritis or corneal opacity, and eyes returned to normal within 1 week. Another difference between the two agents is that CN produces a more severe reaction than CS when applied to the eye in powder form or as a spray at close range (McNamara et al., 1968).

In addition to opacification, corneal effects from particulate CN exposure may include possible penetration of the corneal stroma, melanosis, severe scarring and ulceration, and deficits in the corneal reflex (Blain, 2003; MacLeod, 1969; Scott, 1995). Penetration of the corneal stroma may lead to stromal edema and later vascularization, resulting in further ocular complications. These may include pseudopterygium, infective keratitis, symblepharon, trophic keratopathy, cataracts, hyphema, iridocyclitis, posterior synechia, deformities of anterior chamber angle, secondary glaucoma, vitreous hemorrhage, and traumatic optic neuropathy (Gray and Murray, 1995; Hoffmann, 1967; Leopold and Lieberman, 1971). Furthermore, a 4% CN formulation produced permanent corneal injury but a 10% CS product did not (Gaskins et al., 1972). In animal studies, high concentrations of CN produced ocular necrosis (Grant, 1986).

12.6.1.3 Dibenz(b,f)-1:4-oxazepine

Higginbottom and Suschitzky (1962), who discovered CR, first noted the intense lacrimal response to this compound. A splash of CR (0.01%–0.1% range solution) causes immediate ophthalmodynia, lacrimation, and blepharospasm, similar to CS and CN (Sidell, 1997). These effects can last 15–30 min before subsiding. Blepharitis (edema of the eyelids), periorbital edema, and injected conjunctivae can last for up to 6 h. In rabbits and monkeys, CR (0.1% solution) causes mild transient erythema, chemosis, and keratitis in the eye. Moderate conjunctivitis has been demonstrated with higher CR concentrations (5% solution) applied directly to the rabbit eye (Rengstorff et al., 1975). Ballantyne et al. (1975) showed that increasing CR concentrations as a solution

caused dose-dependent corneal thickening but minor eye effects (mild conjunctivitis and lacrimation) as an aerosol. In animal studies, the effects of CR are very transient and produce far less toxicity to the eye than CN (Salem et al., 2006).

12.6.1.4 Capsaicin

Contact of the eyes with OC causes redness, swelling, pain, tingling, lacrimation, and blepharospasm (Yeung and Tang, 2015). In a retrospective study of 81 patients who presented to the emergency department after aerosol exposure from law enforcement use of OC, 56% of individuals developed ophthalmodynia, 44% developed conjunctivitis, 40% developed conjunctival erythema, 13% developed lacrimation, and 9% developed corneal abrasions (Watson et al., 1996). Kearney et al. (2014) systematically reviewed human exposures to OC recorded into the electronic database of the California Poison Control system during 2002−11. Of the 3671 victims, 1913 (52.1%) reported an ocular route of exposure. Of these, 161 (8.4%) suffered from more severe ocular symptoms that required medical attention, such as persistent pain (more than an hour beyond the completion of a sufficient eye irrigation), reduction of vision, sensation of foreign body, photophobia, discharge or exudate, or periorbital swelling. These symptoms are suggestive of a possible corneal abrasion, iritis, or ocular infection, and persisted from a minimum of 3 h to 5 days. Another study involved exposure of 47 human volunteers to OC for evaluating effects on the cornea and conjunctivae (Zollman et al., 2000). All subjects reported significant eye pain, blurred vision, and lacrimation 10 min after exposure to OC pepper spray, but symptoms improved by 1 h later. Corneal abrasions were not apparent, but 21% of subjects showed evidence of punctate epithelial erosions and reduced corneal sensitivity. Corneal abnormalities were absent 1 week after exposure.

Long-lasting or permanent ocular injuries after OC exposure are rare. Gerber et al. (2011) reported conjunctival proliferation in a young child after OC spray injury which was refractory to steroid therapy. A complete excision of the proliferative tissue was performed 6 weeks after the injury with no recurrence during 2 months of follow-up. Rasier et al. (2015) observed a decrease in tear production in OC victims associated with impaired corneal reflex lacrimation. Additionally, Epstein and Majmudar (2001) reported a case with persisting corneal stromal opacity resulting in irregular astigmatism.

In mice, a single subcutaneous injection of 12.5, 25, or 50 mg/kg capsaicin causes corneal changes characterized by neuronal axon degeneration in the corneal epithelium (Fujita et al., 1984). In rats and rabbits, capsaicin instilled in the eyes (at concentrations up to 100 μg/mL) induces acute irritation, marked disorganization of the corneal epithelia and stroma, and increases opacity, while it decreases corneal membrane permeability, reduces tear secretions and nerve conduction, and increases the expression of inflammatory cytokines (Krishnatreyya et al., 2018).

12.6.2 Nasal/pharyngeal toxicity

RCAs produce oral and nasal symptoms immediately after exposure. Inhalation exposure to CS and CN causes rhinorrhea, sneezing, and burning pain within seconds (Beswick, 1983); a similar burning sensation with increased salivation occurs after oral contact with aerosolized powder or solution. The salivation, pharyngitis, and glossalgia occur within minutes after exposure (Beswick, 1983; Thorburn, 1982). A CR solution (0.01%−0.1% range) splashed in the mouth causes salivation and burning of the tongue and palate for several minutes. Splashes of the solution can cause nasal irritation and rhinorrhea (Sidell, 1997). Fumes from burned *Capsicum* plants or capsaicin-containing pepper sprays are highly irritating to the nasal mucosa and cause immediate rhinorrhea (Geppetti et al., 1988).

12.6.3 Cardiovascular toxicity

RCAs have apparent effects on the cardiovascular system. The response of vessels depends on their localization. For instance, in skin, TRPA1 agonists induce a biphasic reaction associated with vasoconstriction through TRPA1-dependent superoxide production stimulating α_{2C}-adrenoceptors and Rho-kinase-mediated MLC phosphorylation, followed by vasodilation via sensory nerve-derived neuropeptides and neuronal nitric oxide (Aubdool et al., 2014; Pan et al., 2018). Vasodilation has been observed in brain and mesenteric arteries by endothelium-dependent mechanisms (Jin et al., 2019; Pires and Earley, 2018).

Inhalatory exposure to CS in vivo is, however, associated with tachycardia and mild hypertension (Beswick, 1983). Bypassing the pain receptors of the nose and upper airway by endotracheal administration of CS leads to a decrease in blood pressure, effects also seen after intravenous injection. This suggests the initial pressor effect (together with irregular respiration) is possibly a generalized response to noxious stimuli, whereas the bypass causes vasodilation through pulmonary nerve-derived neuropeptides and vagal reflexes. Similar results were found in CR models. Splash contamination of the face or whole-body drenching with dilute CR solution (0.0010% and 0.0025%) causes an immediate increase in blood pressure and bradycardia (Ballantyne et al., 1976). Intravenous administration of CR in cats causes transient but dose-dependent tachycardia. These pressor effects are

postulated to be secondary to CR effects on sympathetic tone to the cardiovascular system or the result of stress and discomfort from the irritation (Ballantyne, 1977; Ballantyne et al., 1977).

TRPA1 agonists have been also shown to have a direct effect on the heart. *TRPA1* activation contributes to Ca^{2+} overload and hypercontraction, worsens myocardial infarction in an ischemia/reperfusion model (Conklin et al., 2019), and increases myocardial dyssynchrony (Thompson et al., 2019). Although Brimblecombe et al. (1972) demonstrated no significant effects of CS on isolated perfused rabbit heart, another report linked exposure of high CS concentrations to the development of congestive heart failure (Hu et al., 1989). Furthermore, underlying cardiac disease has been shown to exacerbate toxicity from CS (Worthington and Nee, 1999).

Capsaicins act on the cardiovascular system largely as agonists of TRPV1. Capsaicin enhances adrenal medullary adrenaline secretion, transiently elevating heart rate and blood pressure in humans (Hachiya et al., 2007). When administered intravenously in rats, a triphasic pressure response is produced, including immediate hypotension, intermediate recovery, and delayed hypotension. Bradycardia occurs at immediate and intermediate phases through stimulation of vagal C-fibers. Vagotomy abolishes immediate hypotension and potentiates the intermediate recovery as hypertensive response (Dutta et al., 2013; Teófilo et al., 2019). A similar triphasic response was found after intravenous injection of nonivamide or nonivamide succinate (Yeh and Chen, 1991). TRPV1 activation is also associated with vasoconstriction of coronary vessels (Szolcsanyi et al., 2001). This is possibly mediated by endothelin release from sensory nerve terminals (Ohanyan et al., 2011). Such a mechanism might explain myocardial infarction (Cil et al., 2012) and two deaths reported in victims with preexisting heart arrhythmia and coronary artery disease following OC exposure (Toprak et al., 2015).

12.6.4 Respiratory toxicity

RCAs are disseminated as an aerosol powder or solution. Therefore, inhalation is a common route of absorption. Inhalation of RCAs causes burning and irritation of the airways, leading to cough, chest tightness, dyspnea (Beswick, 1983; Blain, 2003; Hu et al., 1989), shortness of breath (Euripidou et al., 2004), bronchospasm, and bronchorrhea (Folb and Talmud, 1989).

12.6.4.1 Ortho-chlorobenzylidene malononitrile

According to Dimitroglou et al. (2015), respiratory clinical effects have been reported in 40% of cases after contact with CS. The latency varies from immediate/a few minutes to 2 weeks. Although the symptoms are usually mild and self-limiting, CS has been associated with serious pulmonary complications, including reactive airways dysfunction syndrome, hypersensitivity with pneumonitis and bronchoconstriction, laryngeal obstruction, or laryngospasm.

Laryngospasm can occur immediately or may be delayed for 1−2 days after CS exposure.

Reactive airways are associated with high-level exposure to CS (Blain, 2003). Pulmonary effects typically resolve by 12 weeks after exposure. Roth and Franzblau (1996), however, demonstrated that paroxysmal cough, shortness of breath, and chest tightness, the characteristics of reactive airway disease, may last up to 2 years and can be associated with a need for daily medication.

Pulmonary edema may occur up to 24 h after exposure (Stein and Kirwan, 1964). Delayed-onset bronchopneumonia might occur from prolonged exposure to some RCAs in enclosed spaces (Beswick, 1983). Krapf and Thalmann (1981) reported a 43-year-old man who developed pulmonary edema complicated by pneumonia, heart failure, and hepatocellular damage after contact with CS. Furthermore, in two cases, overexposure to a mix of CS and CN and a mix of CS and OC was the cause of death due to acute necrotizing laryngotracheobronchitis and pulmonary injury, respectively (Toprak et al., 2015).

There is no evidence that CS causes permanent lung damage after one or several exposures to field concentrations (Blain, 2003). Inhalation of an irritant might be expected to exacerbate underlying pulmonary disease such as asthma, emphysema, or bronchitis. Histories of asthma and chronic obstructive pulmonary disease may exacerbate effects from CS (Worthington and Nee, 1999). CS may exacerbate chronic bronchitis or precipitate an attack in individuals with known asthma (Anonymous, 1971). A higher risk of respiratory illnesses was also observed in recruits exposed to CS (Hout et al., 2014).

In animal studies, exposure to aerosol CS (unreported concentration) in male Wistar rats for 20 min can cause decreased minute ventilation and induce histological lesions of the trachea (cytoplasmic vacuoles in epithelial cells) and lung (emphysema) (Debarre et al., 1999).

12.6.4.2 Chloroacetophenone

CN may present symptoms similar to CS. The deaths resulting from prolonged exposure to CN in enclosed spaces were reported to result from pulmonary injury and/or asphyxia due to acute pulmonary edema or necrotizing laryngotracheobronchitis (Blain, 2003; Kim et al., 2016).

In animal models, the cause of death from CN inhalation is also the result of toxicity in the pulmonary system. Postmortem examination from acute toxicity lethality

studies in animals exposed to CN aerosols reveal pulmonary congestion, edema, emphysema, tracheitis, bronchitis, and bronchopneumonia in dogs, and pulmonary congestion, edema, and bronchopneumonia in mice, rats, and guinea pigs (Olajos and Salem, 2001). Sublethal CN aerosol exposure (62.6 mg/m^3, 0.1 LC50) for 60 min causes cellular degeneration in the bronchiole epithelium and alveolar septa thickening attributable to infiltration of mononucleocytes (Kumar et al., 1995).

12.6.4.3 Dibenz(b,f)-1:4-oxazepine

CR does not produce any significant respiratory toxicity (Sidell, 1997). It causes tachypnea and labored breathing in multiple animal species. In humans, aerosol exposure to CR causes respiratory irritation, choking, and dyspnea. One human study involving aerosol exposure to CR (0.25 mg/m^3) in volunteers for 60 min noted decreased expiratory flow rate minutes after exposure. CR was thought to stimulate irritant receptors in the conducting portion of the pulmonary system, causing bronchoconstriction (Ashton et al., 1978). Additionally, CR increased blood volume in the lungs by driving sympathetic tone. Two animal studies evaluated the effect of CR aerosol exposure on the physical and ultrastructural changes in rat lungs (Colgrave et al., 1983; Pattle et al., 1974). Even high CR aerosol doses did not produce significant pulmonary damage. Gross examination of the lungs was normal in both studies. Microscopic examination showed mild congestion, lobar hyperinflation characteristic of emphysema, and hemorrhage. Further pulmonary damage was evident on electron microscopy. CR-exposed lungs showed capillary damage of the endothelium and a swollen epithelial layer.

12.6.4.4 Capsaicin

Minor and self-limiting symptoms, such as cough and transient irritation, were usually observed following OC exposure. Severe adverse effects were observed only in 2.0%–10.7% of cases (Haar et al., 2017; Kearney et al., 2014) and included shortness of breath, chest tightness, and wheezing (Kearney et al., 2014).

In children, capsaicin spray was demonstrated to cause severe bronchospasm and pulmonary edema (Billmire et al., 1996). In the Billmire study, a 4-week-old infant was exposed to 5% pepper spray after discharge from a self-defense device. The infant suffered respiratory failure and hypoxemia requiring immediate extracorporeal membrane oxygenation. Inhaled capsaicin also causes an immediate increase in airway resistance (Fuller, 1991). This bronchoconstriction is dose-dependent (Fuller et al., 1985) and more pronounced in patients with chronic idiopathic cough or asthma (Johansson et al., 2019). While deaths are rare, Toprak et al. (2015) reported several cases related to OC exposure and respiratory failure. The causes of death were associated with asphyxiation due to bronchospasms, acute bronchial asthma, or acute laryngeal edema.

12.6.5 Neurologic toxicity

RCAs are irritants to the peripheral nervous system (COT/COM/COC, 1999), inducing discomfort, burning pain, and associated reflexes. Their neurologic toxicity can range from paresthesias of the lips to burning pain of the eyes (ophthalmodynia), tongue (glossalgia), nose (rhinodynia), throat (pharyngodynia), and skin (dermatalgia). Because RCAs affect the senses, the feeling can become disorienting after exposure, which may explain why some experience temporary loss of balance and orientation after exposure (Thorburn, 1982).

According to Anderson et al. (1996), headache appears to be a common neurologic sign among symptomatic CS victims. Agitation and panic may develop in those not previously exposed to CS and CN (Beswick, 1983; Stein and Kirwan, 1964). Syncope has also been reported (Athanaselis et al., 1990; Thorburn, 1982), but this is likely attributed to panic. When CN was released into 44 prisoner cells, eight inmates experienced malaise and lethargy; among those hospitalized, one experienced syncope and a severe systemic illness (Thorburn, 1982).

A clinical case report of hand injuries caused by accidental discharges from tear gas pens (Adams et al., 1966) revealed specific neuronal toxicological findings. In each case, CN penetrated into the skin to cause a wound. Neurological examination indicated hyperesthesia of select digits in all cases. Biopsies of digital neurons performed for pathology showed thickened epineurium and tendon sheaths. Some of the patients reported paresthesias months after exposure. The study suggests a possible link between direct chemical injury and nerve damage. The same investigators exposed the sciatic nerves of rabbits to the agent by discharge of a CN pen or by dusting the exposed nerve with 0.2 g CN powder. These animal studies suggested that CN can cause inflammation and necrosis in skeletal muscle, loss of axon cylinders, and replacement of neural elements with granulation tissue and fibroblasts (Adams et al., 1966). Animals exposed to CR exhibit fasciculations, tremors, convulsions, and ataxia; intraperitoneal administration of CR can also cause muscle weakness (Salem et al., 2006). These symptoms can be linked with TRPA1 receptors or their function (Nirenberg et al., 2018). In addition, Schwann cells express TRPA1 receptors. Schwann cell TRPA1 mediates neuroinflammation that sustains macrophage-dependent neuropathic pain in mice (De Logu et al., 2017). However, the exact role of TRPA1 in peripheral neuronal damage after RCA exposure remains unknown.

Activation of TRPV1 leads to transitory excitation followed by a prolonged refractory period, indicative of an apparent nonconducting and desensitized state of the receptor. Capsaicin renders the eyes and skin of humans and animals insensitive to various types of painful chemical stimuli (Bernstein et al., 1981). In humans, OC exposure eventually causes loss of the corneal blink reflex (Olajos and Salem, 2001). Furthermore, capsaicin when applied at high doses is an ablative agent for pain-sensing neurons. The mechanism is possibly related to the influx of Ca^{2+} and Na^{+} leading to cell swelling, mitochondrial Ca^{2+} loading, and production of reactive oxygen species (Pecze et al., 2016). Administration of capsaicin in neonatal rats causes destruction of the dorsal root ganglion neurons (Jancsó et al., 1977).

Concerns have been raised about the psychiatric effects of RCAs. Unuvar et al. (2017) explored psychiatric morbidity in a group of individuals seeking medical care for injuries after the 2013 Gezi Park protests in Turkey. The police reported that they mostly used OC and CS as RCAs. Psychiatric evaluations were conducted in 117 cases. Of these, 83 (71%) met the diagnostic criteria for acute stress disorder, posttraumatic stress disorder, or major depressive disorder.

12.6.6 Gastrointestinal toxicity

In a systemic review covering 90 cases, gastrointestinal effects were reported in 13% of cases after contact with CS (Dimitroglou et al., 2015). Salivation, nausea, vomiting, anorexia, abdominal pain, diarrhea, hematemesis, mouth ulcers, and alterations in taste have been reported in clinical case reports of exposure to CS (Anderson et al., 1996; Athanaselis et al., 1990; Solomon et al., 2003) and CN (Blain, 2003; Thorburn, 1982). The involvement of retching and emesis tends to occur if the individual is sensitive, the concentration is sufficiently high, the exposure is prolonged, the range is close, or the event occurs in a confined space. Vomiting was reported in 25% of patients with severe reactions to CN in a confined area (Thorburn, 1982). Emesis did not resolve until the following week in one patient. Inhalation of RCAs often leads to parageusias or altered taste of the tongue. In particular, a metallic or burning sensation is often reported (Folb and Talmud, 1989).

Ingestion of CS can also produce episodes of nausea, vomiting, crampy abdominal pain, and diarrhea (Blain, 2003; Solomon et al., 2003). Seven patients in the Solomon study drank juice contaminated with CS pellets and primarily developed gastrointestinal symptoms. Two of the seven patients reported emesis and diarrhea; all patients reported abdominal pain, epigastric discomfort, and burning gastroesophageal reflux. Symptoms resolved 24 h later. Surprisingly, they did not develop parageusia or burning of the tongue after CS ingestion, which is often the case after inhalational CS exposure. Another study designed for patients to taste an admixture of sugar and CS (5−10 pellets, 500 mg each, dissolved in 10 L of water) indicated that patients experienced a 30 s delay in onset of altered taste (Kemp and Willder, 1972); this was most likely because of a masking effect from the sugar. In animal studies, rabbits and rats develop gastroenteritis on CN or CS exposure by ingestion (Gaskins et al., 1972).

Gastrointestinal symptoms have been documented also following contact with OC. According to the electronic database of the California Poison Control system during 2002−11, ingestion was the primary route of exposure in 4.2% of cases. Severe symptoms that required medical attention were extremely rare (0.05%) (Kearney et al., 2014). The symptoms usually include mouth irritation, nausea, and vomiting (Ballantyne, 2006). Nausea has also been reported in individuals exposed to pepperball tactical powder containing capsaicin (Hay et al., 2006). Capsaicin causes effects on gastric mucosa, including mild erythema, edema, epithelial cell damage, and gastric hemorrhage (Desai et al., 1977).

12.6.7 Dermatological toxicity

RCAs are primary irritants of the integumentary system. Skin can be sprayed directly. However, hours after dissemination, CS, CN, or CR can pose a toxic danger because they are persistent in the environment. During the riots of the late 1960s, CS was frequently used to control crowds. Inadvertently, firefighters in those metropolitan areas sometimes were exposed as they entered buildings where CS had been disseminated. The force of water from fire hoses and movement within the buildings reaerosolized enough agent to cause erythema and edema around the eyes and other areas of exposed skin (Rengstorff and Mershon, 1969a). In 2003, a number of staff working in a retail store in Scotland became ill after the delivery of furniture exposed to CS, which was used to detect unauthorized stowaways aboard a vehicle (Hankin and Ramsay, 2007).

12.6.7.1 Ortho-chlorobenzylidene malononitrile

According to Dimitroglou et al. (2015), dermatitis is described in 61% of cases following contact with CS. The latency varies from immediate/a few minutes to 2 weeks depending on the symptom. Low concentrations cause erythema, pruritis, subcutaneous edema, paresthesias, and/or burning sensations in exposed areas of the skin within minutes. Burning sensation and erythema are often the first signs of contact dermatitis, subsiding approximately 1 h after exposure. These agents follow a time course of

skin damage similar to that of mustard agent. Further, if the skin is wet or abraded, then the toxic effects on the skin are more prominent (Holland and White, 1972; Sidell, 1997; Thorburn, 1982). Exposure to higher doses leads to worsening erythema, edema, vesication with bullae (observed hours later), and fever. Typically, edema and vesiculation (bullae dermatitis) occur 24 h after CS exposure (Sidell, 1997). Ongoing skin contact can cause wound overgranulation, hypertrophic scarring, or burns requiring surgical debridement and grafting (Agrawal et al., 2009).

The extent of toxic effects also depends on the thickness of the stratum corneum and time of exposure. Furthermore, contact with water up to 48 h after exposure can exacerbate the painful symptoms (Blain, 2003; Pinkus, 1978). High humidity, diaphoretic subjects, and warm temperatures can all exacerbate the contact dermatitis from RCAs (Hellreich et al., 1969). Areas of occlusive dress over the skin may also result in worse reactions. Common sites of bullae are areas under the cuff of a shirt or glove and just under the collar. One study examined the effect of high CS concentrations (300 mg/m^3) tested on the arms of volunteer study patients for exposure times ranging between 15 and 60 min (Hellreich et al., 1967). All participants experienced burning pain approximately 5 min after exposure onset. A Ct range of 4440−9480 mg min/m^3 caused immediate patchy erythema, which subsided after 30 min. A Ct range of 14,040−17,700 mg min/m^3 led to greater dermal toxicity and required several hours to subside. Bullous dermatitis occurred in 50% of subjects as a delayed reaction. These bullous lesions resolved in 2 weeks, but an inflammatory hyperpigmentation of the skin remained 6 weeks after exposure. Differences in individual sensitivities are attributable to skin pigmentation, complexion, and susceptibility to sunburns (Hellreich et al., 1969).

Dermal exposure to CS may lead to allergic contact dermatitis (Watson and Rycroft, 2005), a delayed hypersensitivity reaction that develops from a previous exposure to RCAs. Initial exposure to CS may not cause significant toxic signs or symptoms. Exposure to small amounts of the same agent years later, however, it may produce a severe allergic erythematous, patchy rash with edema, bullae, purpura, and necrosis. Sensitization is likely to occur after dermal exposure to high concentrations of RCAs (Holland and White, 1972). Hypersensitivity reactions can persist for up to 4 weeks. This phenomenon has been demonstrated so far by CN (Frazier, 1976; Ingram, 1942) and CS (Ro and Lee, 1991), but not CR.

12.6.7.2 Chloroacetophenone

CN is a more potent irritant than CS. In a human study involving dermal application, CN (0.5 mg) powder caused irritation and erythema when applied on the skin for 60 min (Holland and White, 1972). It took 20 mg CS to cause similar effects for the same duration of exposure. CN is also a more potent sensitizer (Chung and Giles, 1972).

12.6.7.3 Dibenz(b,f)-1:4-oxazepine

Dermal exposure to CR causes a burning sensation and erythema several minutes later. The burning pain goes away after 15−30 min, but the erythema lasts up to 2 h (Holland, 1974). CR does not induce inflammatory cell migration to the site of injury, bullous dermatitis, or contact sensitization (Ballantyne et al., 1977). Repeated application of CR to the skin (applied 5 days/week for 12 weeks) has little effect (Marrs et al., 1982). Similarly to the eye and lungs, CR does not demonstrate significant toxicity to the skin.

12.6.7.4 Capsaicin

A dermal route of exposure was observed in 59.5% of cases. Severe symptoms, such as rash and blisters, are present in 2.8% of exposed individuals (Kearney et al., 2014). Symptoms usually involve immediate and severe erythema and edema in the skin (Herman et al., 1998). Similarly, pepperball pellets fired at individuals will cause erythema, pain, and edema at the site of impact. The initial point of contact may become infected, scar, or heal with hyperpigmentation (Hay et al., 2006). Although capsaicinoids may have a vesicant effect, depending on the length of exposure, in most cases it produces only a burning sensation and mild erythema (Herman et al., 1998; Watson et al., 1996). Skin blistering and rash may occur after chronic or prolonged capsaicin exposures. Capsaicin can cause allergic contact dermatitis (Lambrecht and Goossens, 2015).

12.6.8 Other toxicity

One report noted renal tubular nephritis in a worker killed after an explosion inside a plant manufacturing CS agent (Cookson and Nottingham, 1969). Hepatocellular injury has been linked to serious CS inhalation (Krapf and Thalmann, 1981). To date, animal studies have not documented any relationship between RCA exposure and teratogenicity (Folb and Talmud, 1989; Upshall, 1973). CS did not demonstrate mutagenic potential with the Ames assay (Rietveld et al., 1983) and lacks mutagenicity in several test systems (Wild et al., 1983). Ames assay did not reveal any genetic toxicity of CN (NTP, 2018). CR did not have carcinogenic effects in mice or hamsters (Blain, 2003). In the case of capsaicin, both positive and negative effects have been found in classical genetic toxicology assays. Genotoxicity seems to be absent in studies

conducted with high-purity capsaicin, even with metabolic activation. Therefore, it is possible that the genotoxic potential observed in some of the studies was associated with other compounds or mutagenic impurities in the test compounds (Bley et al., 2012; Tsuchiya et al., 2011).

12.6.9 Lethality

Human deaths have been reported from RCA exposure. Estimates of the human LCt50 are presented in Table 12.2. The large safety ratio for CR is clearly evident as compared with the other agents, including OC and PAVA (Satpute et al., 2018). According to the study by Toprak et al. (2015), which analyzed 10 cases of lethal exposure to RCAs, death occurs between 18 and 22 min and 4 days following the initial contact. Death is usually the result of excessive concentrations used, confined spaces, and prolonged exposures. Not surprisingly, several cases involved prison inmates or a subject who barricaded himself in his room after struggling with the police and was exposed to CN in this room for approximately 30 min with limited ventilation (Thorburn, 1982; Toprak et al., 2015). Preexisting cardiovascular and/or pulmonary problems can be factors contributing to death. Drug use may also increase RCA toxicity. This was demonstrated by the combination of cocaine and capsaicin in mice (Mendelson et al., 2010).

12.6.10 Traumatic injuries

Although rare, mechanical trauma can occur when RCAs are deployed. Traumatic effects usually arise from direct targeting of individuals, both with the projectile canister as well as spray to the face (Haar et al., 2017). Severe maxillofacial injuries were observed (Çorbacıoğlu et al., 2016). Alhillo et al. (2018) reported a case of a tear gas-induced direct traumatic injury to the neurocranium. The energy density of the gas jet and the high temperatures of the exploding gas volume cause extensive soft tissue damage and burns, which in this case resulted into the patient's death 1 h after the admission. The largest series of traumatic injuries was reported from India on 18 patients with vascular injury caused by tear gas shells (Wani et al., 2011). Of these, six cases (33%) were associated with nerve injuries, four (22%) with skeletal fractures, and three (17%) resulted in amputations.

RCAs may also spark mass panic and stampedes that contribute to significant morbidity and mortality, particularly in enclosed spaces without safe avenues (Haar et al., 2017). This includes a case of at least 17 deaths in a club in Venezuela in 2018 due to a tear gas canister, causing panic among the hundreds of visitors (Casey, 2018).

12.7 Risk assessment

The ideal process in RCA risk assessment is to characterize the effectiveness and risk from exposures to situations in which RCAs may be used (NAS/NRC, 1994; Patterson et al., 2004; TERA, 2001). To do that, one must identify all pertinent effects of the RCA in question, develop a dose–response assessment, consider an exposure assessment, and finally characterize the risk. When used as intended, RCAs are thought to be safe and of sufficiently low toxicity. However, they are not without additional unwanted effects, especially in circumstances in which high concentrations are used or exposure is prolonged. The previous sections have provided sufficient discussion regarding the potential toxicity to humans as a result of exposure to RCAs, including case reports.

12.7.1 Identification of intended and unintended effects

By providing a minimal force alternative for controlling and managing individuals, RCAs are a desired public health and safety tool for military, domestic law enforcement, and civilian use. As with any chemical intended to benefit the public, it is important first to identify the compounds, their potential adverse impact (unintended effects), and their beneficial impact (intended effects). There are a number of chemicals designed and used as RCAs. In general, they are compounds with low vapor pressures and dispersed as fine particles or in solution from a variety of devices. These dispersal methods can include the gamut from aerial spray (Fig. 12.6) to large spray tanks (see Figs. 12.2 and 12.4) and small hand-held devices for self-protection. The modern RCAs used today include CN, CS, CR, OC, and PAVA. Their major adverse effects are summarized in Table 12.2. The intended effect for all RCAs is a change in behavioral response of the target. A better measure of this intended effect would be the actual physiological effects produced by RCAs on the eyes, skin, and respiratory tract (Patterson et al., 2004). These are the target organs designed for irritation by RCAs (see Fig. 12.11 for review).

12.7.2 Dose response

Dose–response assessment involves evaluating the dose required to produce a particular effect of interest. Ideally, quantitative data on specific doses and their corresponding responses are desired. In reality, threshold data for a particular target organ or effect in a target organ are often available as a substitute. The ophthalmic threshold levels and toxicity estimate for human responses to CN, CS, and CR are shown in Table 12.2. If empirical dose–response

data are available, then a dose–response evaluation for a given RCA might include plotting the percent of individuals responding as a function of dose for each toxicological sign or symptom and target organ of interest. Dose–response curves can then be used in modeling studies to estimate the probabilities of intended and unintended effects for a particular risk assessment scenario (Patterson et al., 2004).

12.7.3 Exposure assessment

The crux of exposure assessment is creating a scenario for human exposure to a given RCA and identifying the exposure factors. This would involve describing the intended targets, environmental conditions (windy, rainy, hot weather, etc.), crowd size and characteristics, delivery device (tear gas canisters or grenades, powder or aerosol), hazards associated with the delivery system such as blunt trauma, the nature of the agent selected (physicochemical properties, solvents, concentration/dose), and duration of exposure. An exposure assessment might include estimation of the amount of systemic exposure through RCA inhalation, absorption through the skin from dermal contact, or intestinal uptake after ingestion. Availability of quality data for each of the aforementioned exposure factors will estimate exposure with high confidence and a minimal uncertainty level. Unavailability of data is a major limitation if models are used to estimate exposure.

12.7.4 Characterization of the risk and risk management

Estimating or developing probabilities of toxic effects within a population is at the heart of risk characterization. It integrates dose–response and exposure assessments. It is designed to provide the probability of occurrence for effects induced by a given RCA given a particular exposure scenario. For example, a decision-maker will use risk characterization to estimate the probability of a group of effects occurring as a result of clearing a confined space with CS. The probability can be derived as a function of the number of tear gas grenades used. Unfortunately, there is a dearth of specific federal risk assessment and risk management guidance or mandates on RCAs. Therefore, the potential for risk management or mitigation of concerns is not optimized for the health and benefit of the public good (Hauschild et al., 2004). Standardization of the process for assessing risk of toxic chemicals (Burke, 1993) and computer modeling with empirical can be a powerful predictor for risk assessment of any toxic chemical.

12.8 Treatment

Exposure to RCAs leads to a generalized stress reaction, causing leukocytosis (Thorburn, 1982), hypokalemia, hyponatremia, hypochloremia, elevated total protein, increased globulin, and high bicarbonate levels (Beswick et al., 1972). Treatment for RCA toxicity is not often required because the course of intoxication is self-limiting for the most part. Clinical signs and symptoms from RCA exposure subside in less than an hour. Initial care involves removing the victim from a potentially crowded area of dispersal immediately to minimize exposure time. It is important to note that these victims may require additional assistance during evacuation because of their reduced vision and disorientation. In circumstances in which the concentration of the agent is substantially elevated or the area of release is confined, increased complications and risks of morbidity may arise in the eyes, skin, airways, and lungs.

12.8.1 Eyes

If the eyes are involved to any degree, then a protective mechanism to close the eyelids will be initiated as a result of conjunctivitis, iritis, or keratitis. Photophobia, blepharospasm, and apraxia of eyelid opening prevent the clinician from evaluating the damage. However, a local anesthetic applied to the eye will help with eye pain and allow for further evaluation of the eye by slit lamp. Contact lenses should be immediately removed and the eyes flushed of any dusting or agglomerated solid particles (see Fig. 12.12). Eyes should be irrigated with copious volumes of water or saline for at least 15 min to adequately flush the irritant. The irrigation liquid must be cold as otherwise symptoms can worsen (Svinos, 2011). Diphoterine has been used for eye and skin decontamination after CS exposure (Viala et al., 2005). Boric acid wash is contraindicated (Kim et al., 2016). If symptoms or signs of toxicity persist, consultation with an ophthalmologist is critical because there is a possibility of embedded particles that need removing. Elderly patients should be monitored for evidence of possible acute glaucoma (Yih, 1995).

12.8.2 Skin

Early signs of skin toxicity at the time of clinical presentation will often be contact or allergic dermatitis because blisters form hours later. Removal of clothing should be the first step in decontamination. Placement of contaminated clothes in sealed plastic bags by first responders will prevent secondary contamination as a result of reaerosolized agent (Horton et al., 2005). Early studies of CS indicated that mixing CS with sodium hypochlorite

(or household bleach) produced a greater reaction than CS alone in patch testing (Punte et al., 1963). Despite its usefulness as a decontaminant for many chemical agents, hypochlorite should never be used to decontaminate RCAs on skin. Use of water for decontamination of skin may result in an initial worsening of the burning sensation (described previously). A solution of 6% sodium bicarbonate, 3% sodium carbonate, and 1% benzalkonium chloride has been shown to provide immediate relief from CS dermatitis as the alkaline solution hydrolyzes the agent (Sidell, 1997; Weigernd, 1969). Consultation with a burn unit should be considered when large areas of skin are involved or when children are affected. Medical treatment for dermatitis may include topical steroids such as triamcinolone acetonide, oral antihistamines for pruritis, and topical antibiotics such as silver sulfadiazine (Hellreich et al., 1967; Sidell, 1997). Systemic antibiotics can be administered for secondary infection. Oozing lesions from bullae dermatitis should be treated with wet dressings and changed daily. Deroofing closed vesicles is controversial (Carvajal and Stewart, 1987). Tetanus prophylaxis should be considered.

12.8.3 Respiratory

Removing an exposed patient from the source of intoxication to fresh air will provide immediate improvement. Patients should be evaluated for hypoxia with pulse oximetry and arterial blood gases. Pulmonary function tests may be helpful in patients with prolonged pulmonary symptoms and followed-up until symptoms resolve. Chest radiography might be useful if the concentration was sufficiently high, exposure was prolonged, or dispersal occurred in a confined space. Pulmonary edema may be delayed for 12–24 h after exposure, suggesting a need for follow-up radiographs (Solomon et al., 2003; Stein and Kirwan, 1964). Laryngospasm is a serious complication that may require tracheal intubation to secure a patent airway. Bronchospasm may be treated with inhaled beta-2 agonists, steroids (methylprednisolone), and aminophylline (Ballantyne and Swanston, 1978; Folb and Talmud, 1989). Arterial blood gas and pulse oximetry should be continued if patients are symptomatic hours after exposure.

12.9 Concluding remarks and future directions

The goal of RCAs is to irritate or produce temporary incapacitation. Law enforcement agencies and military personnel use RCAs for quelling protestors, controlling crowds, subduing combatants, clearing buildings, training in chemical warfare, and area denial. Individuals use hand-held devices for self-protection against an assailant. RCAs are dispersed as aerosols or sprays, causing irritation of mucous membranes of the eyes, respiratory tract, and skin. Symptoms and signs of toxicity typically subside by 30–60 min.

Several lines of evidence suggest that RCAs are safe if used as they were originally intended. Even though RCAs are considered safe, nonlethal, temporary incapacitating agents, they are not without risk. Some of the adverse clinical effects from RCA exposure reported in the literature have involved indiscriminate use (excessive concentrations), prolonged exposure, and dissemination of compounds in a confined space. In summary, these nonlethal agents can pose a serious health hazard in their intended targets. Some RCAs have such a poor safety profile that they were abandoned long ago (DM and CA). CN and CS have a large body of literature from which to compare and contrast their safety, toxicity, and potency. As the data clearly suggest, CS is a safer compound compared with CN. The latest newcomers to the RCA scene are the inflammatory capsaicinoids. OC and PAVA are highly effective irritants that cause similar symptoms as CN and CS. Capsaicinoids gained considerable attention in the 1990s from police departments and the public at large for safe and effective chemical incapacitation of individuals. Although OC and PAVA produce a similar constellation of toxic signs and symptoms, little is known about PAVA. More research will be required to determine whether it is safe for humans.

Another gap is opening for possible future use of TRPA1 and TRPV1 antagonists. Although TRPA1 and TRPV1 antagonists are not generally available, several have been undergoing clinical trials. They are expected to be optimal for attenuating pain and other neuroinflammatory-related conditions. Such compounds may however significantly affect protective reflexes as well as pain-related behavior, resulting in excessive exposure to RCAs. Research starting with dose–response assessment in animal models testing RCAs together with TRPA1 or TRPV1 antagonists at clinically relevant concentrations up to epidemiological studies correlating the use of antagonists with the occurrence and severity of adverse effects will be necessary to help decision-makers on future utilization of RCAs.

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Chapter 13

Phosgene oxime

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13.1 Introduction

Amongst the several chemical warfare agents manufactured or developed, vesicating agents comprise of chemicals like mustard agents sulfur mustard [SM; bis(2-chloroethyl) sulfide, HD]; nitrogen mustards [HN1 (bis(2-chloroethyl) ethylamine), HN2 (2,2′-dichloro-*N*-methyldiethylamine), and HN3 (tris(2-chloroethyl)amine hydrochloride)]; and arsenical vesicant lewisite [L; LEW; dichloro(2-chlorovinyl) arsine] that cause blistering in addition to acute and debilitating injuries to several organs (Young and Bast, 2009). Alkylating mustard vesicating agent SM has been most commonly used as a chemical agent of warfare and terrorism that causes painful skin blisters and injury largely to the skin, eyes, and respiratory system, and also systemic toxicity (Balali-Mood and Hefazi, 2005; Ghabili et al., 2010, 2011; Layegh et al., 2015). Phosgene oxime (dichloroformoxime; Cl_2CNOH), grouped with vesicating agents, is a halogenated oxime, urticant, nettle, or corrosive agent and does not cause blisters (Augerson, 2000; McManus and Huebner, 2005; Bartelt-Hunt et al., 2006; Patocka and Kamil, 2011). The military designation of phosgene oxime is "CX." Upon exposure CX causes intolerable pain, intense itching, and a rash similar to hives, leading to violent effects with severe tissue damage upon exposure (Augerson, 2000; Patocka and Kamil, 2011; Tewari-Singh, 2018). Although use of CX in warfare has not been reported, CX was first produced in Germany in 1929 and is reported to have been developed by both Germany and Russia and stockpiled during World War II as a potent chemical weapon. CX penetrates clothing and rubber much more quickly than other chemical agents; therefore, could be used alone or with other chemical warfare agents to cause startlingly rapid incapacitation, injuries, and death (Augerson, 2000; Patocka and Kamil, 2011). The intense pain from CX can cause removal of protective masks and clothing and its corrosive effects might render human skin more vulnerable to penetration by other chemical agents such as nerve agents. The extreme irritation and instant eye damage and future lung injury are also of high military significance.

There are suggestions of Iraqi use of an agent whose effects resembled CX against Iran, but there is no confirmation of this use. More recently, in a surprise report [March 20th 2019 (News9.com)], an FBI investigation found chemical warfare agent inside a Lawton home in Oklahoma, USA; testing uncovered that it was CX (https://www.news9.com/story/40170458/fbi-investigation-finds-chemical-warfare-agent-inside-lawton-home). Its easy synthesis makes it a dangerous chemical with both military and terrorist potential. CX penetrates clothes and rubber much more rapidly than other chemical agents and causes quick onset of severe and prolonged effects, including skin lesions that are comparable to those caused by a strong acid (Augerson, 2000; Patocka and Kamil, 2011; Tewari-Singh et al., 2017). CX is the most potent and dangerous chemical threat agent categorized with the vesicants; however, it is the least studied categorized vesicating agent and its mechanism of action is not fully understood, with no specific antidote available (Augerson, 2000).

13.2 Properties and chemistry

CX (Cl2C═N−OH, CAS RN 1794-86-1; Table 13.1) is a halogenated oxime and a colorless, crystalline solid at temperatures below 95°F with a strong, disagreeable odor and violently irritating vapor (Patocka and Kamil, 2011; Ubels et al., 1982). As a munitions ranking compound, CX is in liquid form, with a yellowish-brown appearance. Prismatic crystals of CX are hygroscopic and have a melting point of 39°C−40°C, and a boiling point of 128°C at 76 mm of Hg. It has a high vapor pressure (11.2 mmHg at 25°C) and could easily vaporize at ambient temperature (c20max = 21 g/m^3). CX can possess high mobility in soil

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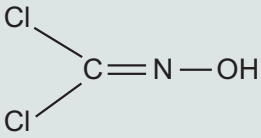
TABLE 13.1 Phosgene oxime.

Synonyms	Dichloroformoxime; CX
CAS No.	1794-86-1
Chemical formula	$CHCl_2NO$
Chemical structure	

based on an estimated adsorption coefficient K_{oc} of 68. It can partially exist as an anion in the environment as it is a weak acid with an estimated p*K*a of 6.5. It is soluble in water and organic solvents (alcohol, ether, and benzene) and hydrolyzes very quickly, predominantly in the presence of an alkali to form hydrogen chloride and hydrolamines. Its hydrolysis at an unspecified half-life and temperature is reported to be 83 days. It decomposes in contact with most metals as it is also corrosive to most metals. CX possesses a very unpleasant and irritating odor and is also very unstable, reactive, and volatile (1800 mg/m^3 at 20°C). CX is heavier than air and thus settles in low-lying areas but does not last in the environment for long. CX can be synthesized by the reduction of chloropicrin with tin in the presence of hydrochloric acid (Patocka and Kamil, 2011). The physical and chemical properties of CX are summarized in Table 13.2. It can be mixed with other chemical threat agents, such as SM, LEW, and nerve agents, and rapid skin damage from its exposure makes the target more susceptible to the second agent exposure. CX is grouped together with vesicating agents, however, it is an urticant or nettle agent and not a pure vesicant as it does not lead to blister/vesicle formation (Tewari-Singh et al., 2018). The nature of injuries caused by CX resembles those caused by urticants or acids, therefore it is often referred to as a nettle or corrosive agent (Patocka and Kamil, 2011).

TABLE 13.2 Physicochemical properties of phosgene oxime.

Property	Phosgene oxime
Physical state	Colorless, crystalline solid or yellowish-brown liquid (munitions-grade); solid form can sublime
Molecular weight	113.93 Da
Boiling point	128°C (760 mmHg)
Melting point	35°C–40°C
Solubility	Readily soluble in water (70% solubility); very soluble in organic solvents
Vapor density (compared to air)	<3.9
Vapor pressure	11.2 mmHg at 25°C (solid) and 13 mmHg at 40°C (liquid)
Volatility	1800 mg/m^3 (20°C)
Decomposition temperature	<128°C
Odor	Disagreeable, prickling odor

13.3 Exposure and toxicity

Toxicity upon exposure to chemical warfare agents like vesicants varies extensively depending on the dose, route, and form of its exposure. CX occupational exposure could be infrequent as there are no industrial uses of this compound similar to other vesicating agents like SM and L, it has only been reported as a chemical warfare agent. Exposure to vesicating agents leads to ocular, skin, and pulmonary damage even at low doses, while higher dose exposures cause multiorgan toxicity (Augerson, 2000). CX exposure in liquid or vapor form can cause more severe damage to the skin, eyes, and lung tissues than other vesicating agents due to its fast penetration (it can even penetrate rubber gloves and clothing) but has not been well studied. This leads to instant pain, tissue destruction, skin damage, severe systemic toxicity, and rapid mortality (Augerson, 2000).

It is reported that within seconds of exposure to low doses of CX (0.2 mg min/m^3), skin and pulmonary membrane irritation begins, and unbearable pain and irritation can start minutes after exposure to a dose of 3 mg min/m^3. Lethal systemic dose of CX estimation [LCt50 (concentration-time product capable of killing 50% of exposure areas)] is 1500–2000 mg min/m^3 (Ubels et al., 1982; Costagliola et al., 2013). People could be exposed to CX mainly via cutaneous, ocular, or pulmonary routes with local tissue destruction. If liquid CX contaminates water or food, people can be exposed by drinking water or eating contaminated food (Patocka and Kamil, 2011).

The rapid cutaneous injury instigated by CX makes the skin vulnerable to damage from other chemical agents. The LD50 for CX cutaneous exposure is estimated as 25 mg/kg. At higher concentrations or increased skin exposure time, CX causes more damage and triggers instant pain followed by tissue necrosis, systemic effects, and mortality (Augerson, 2000). After instant dermal absorption, CX exposure results in immediate itching, pain, skin blanching, erythema, edema, and hives

formation (Tewari-Singh et al., 2018). These lesions can be extremely painful and immediate, resembling stinging nettle with a blanched (white) exposed skin area surrounded by an erythematous ring (Patocka and Kamil, 2011; Tewari-Singh et al., 2017). Within minutes to an hour, the exposed area is edematous, which resolves within a day with dark pigmentation and severe necrosis. With time, more severe desquamation with necrosis of the skin could be observed, followed by eschar formation and polymorphonuclear infiltrates with intense inflammation, with healing taking weeks to months (Augerson, 2000; Tewari-Singh et al., 2017). Severe CX cutaneous exposure could result in pulmonary edema, other systemic effects, and mortality; however, long-term effects are unidentified (Augerson, 2000; Patocka and Kamil, 2011; Tewari-Singh et al., 2017).

Immediate eye and respiratory irritation upon CX vapor exposure causes cough, throat pain, increased lachrymation, and impaired vision (Augerson, 2000). Eyes are reported as the most sensitive organ to vesicating agent exposure (Gordon et al., 2009; Kadar et al., 2009, 2013a,b; Ghasemi et al., 2013; McNutt et al., 2012; Goswami et al., 2016a,b; Tewari-Singh et al., 2016). Ocular CX exposure results in instant and severe pain, irritation, edema, lacrimation, conjunctivitis, and blepharospasm. Severe or long-term CX exposure can result in keratitis, iritis, corneal perforation, and blindness (Patocka and Kamil, 2011). In contract to SM, there are no documented reports on the long-term ocular effects of CX. Upon inhalation, CX can be rapidly absorbed, triggering instant and incapacitating irritation, pain, runny nose, hoarseness, as well as local tissue obliteration of the upper airways at low exposure doses. At higher CX exposure doses, serious complications such as pulmonary edema followed by tachypnea, dyspnea, and cyanosis can transpire (Ubels et al., 1982; Augerson, 2000; Patocka and Kamil, 2011). Exposure to CX aerosol can cause necrotizing bronchiolitis and pulmonary edema with pulmonary vein thrombosis (Augerson, 2000). Like ocular complications, long-term respiratory effects of CX are unidentified, although it is believed to result in pulmonary fibrosis (Augerson, 2000).

In addition to toxic effects to the eyes, respiratory, and skin systems, CX exposure can also cause severe and life-threatening injury to internal organs as reported from our recent study (Tewari-Singh et al., 2017). CX cutaneous exposure resulted in dilatation of the peripheral vessels (including capillaries and sinusoids) and the pooling of red blood cells (RBCs) in the vessels of the lung, liver, spleen, kidneys, and heart tissues (Tewari-Singh et al., 2017). This severe vascular dilation and perhaps leakage could result in a marked loss of blood from the vessels into the adjacent tissue and could lead to low blood pressure, relative hypoxia, and shock, leading to mortality. This could be similar to the "lewisite shock" effect, where death may result from fluid loss, hypovolemia secondary to capillary leakagefrom high-dose exposure of LEW (Snider et al., 1990; Watson and Griffin, 1992).

13.4 Mechanism of action

Among vesicants, molecular mechanisms of SM (Dacre and Goldman, 1996; Kehe et al., 2008; Shakarjian et al., 2006, 2010; Mouret et al., 2015) and LEW (Goldman and Dacre, 1989; Augerson, 2000; Kehe et al., 2009; Nguon et al., 2014; Li et al., 2016; Srivastava et al., 2016) toxicity have been extensively studied. Unlike vesicating agents like SM and LEW, studies and reports on the acute toxicity, long-term effects, and related mechanisms from CX exposure are unidentified (Augerson, 2000).

It is possible that CX possesses alkylating and nucleophilic properties like vesicants or its effects are due to its breakdown products like chlorine, oxime, or carbonyl groups. Its toxicity could be direct, involving corrosive injury, cell death, and tissue destruction, or indirect (involving activation of alveolar macrophages, recruitment of neutrophils, and release of hydrogen peroxide) including an inflammatory response which could lead to delayed tissue injury and pulmonary edema (Augerson, 2000; Tewari-Singh et al., 2017). To study the pathophysiology of acute CX cutaneous exposure, we have recently conducted comprehensive research in SKH-1 hairless mice. Immediate increases in skin injury parameters, including skin bi-fold thickness, erythema and edema, and necrosis was observed. These studies indicate that the toxicity response and skin urticaria from CX resembles the anaphylactic reaction and urticaria from allergic reactions, involving an inflammatory response mainly due to mast cell activation. Data from our published (Tewari-Singh et al., 2017) and completed studies (data not published) in SKH-1 hairless mice show that CX cutaneous exposure causes mast cell degranulation and release of mediators including histamine and tryptase, proinflammatory cytokines, and an inflammatory response in the skin tissue that is associated with edema, erythema, necrosis, urticaria, and blanching. CX cutaneous exposure in mice also caused vasculature dilation and blood congestion in multiple organs, resulting in systemic toxicity, decreases in breath and heart rate, temperature drop, and mortality. These symptoms are similar to anaphylaxis (potentially lethal multisystem allergic reaction with acute respiratory and cardiovascular compromise leading to unconsciousness, shock, and mortality), which is a result of sudden systemic release of mediators from mast cells. There are reports that neutrophil infiltration could also play a key

role in skin inflammation related to mustard vesicating agents (Wormser et al., 2005; Shakarjian et al., 2010; Jain et al., 2014).

In the skin tissue, DNA damage, and p53 phosphorylation and accumulation have also been shown to play a significant role in vesicating agent-induced cell death and injury (Kehe and Szinicz, 2005; Paromov et al., 2007; Goswami et al., 2016a,b). Similar to mustard vesicating agents, published reports from our studies indicate that p53 phosphorylation and its accumulation in skin tissue could be important consequences of CX-induced skin injury. Cutaneous CX exposure also resulted in TNF-α and COX-2 level increases in the skin tissue, paralleling reports of exposure of the skin tissue with other vesicating agents (Shakarjian et al., 2010; Tewari-Singh et al., 2009, 2017). Cutaneous exposure to mustard vesicating agents at higher doses results in damage to multiple organ systems, but mortality is rare (Dacre and Goldman, 1996; Kehe et al., 2008; Patocka and Kamil, 2011; Goswami et al., 2015). However, our studies indicate that CX is absorbed instantaneously, leading to more severe systemic toxicity and mortality compared to other vesicating agents.

These recent studies suggest that further examination to dissect the mechanism of action of CX from its exposure are warranted, mainly to investigate if mast cell activation and associated release of mediators are the major events following CX cutaneous exposure. Such studies are being carried out in our laboratory to identify the signal transduction pathways and novel targets for therapeutic intervention to mitigate CX-induced morbidity and mortality.

13.5 Protection, decontamination, and treatment

If there is an exposure to CX, moving immediately upward and toward an area where fresh air is available is an important protection since CX is heavier than air and settles down (Patocka and Kamil, 2011). Pressure-demand, self-contained breathing apparatus (SCBA) could be helpful in situations of CX exposure. Although CX can attack butyl rubber gloves and boots, these are protective against field concentrations of CX. Since CX is absorbed very rapidly, skin and eye decontamination are needed immediately; hence, the timing of decontamination is very critical. CX reacts with tissues very rapidly and causes extreme pain and itching—if this occurs then decontamination will have no effect. All alkaline agents can be used for chemical inactivation. Chlorinated agents cannot be used for CX decontamination and water can only be used to flush the chemical from eyes. Clothing exposed to CX should be immediately removed and sealed in a bag to avoid any further exposure and contamination. Immediate and lifesaving care might be required within minutes following CX exposure at an emergency station. Long-term care, hospitalization, and lifesaving surgery can be required, and delay in this care can adversely affect the injury and survival outcomes.

There is no effective antidote available against CX-induced toxicity and any treatment is mostly helpful to reduce indications, prevent infections, and help healing. Systemic analgesics are a better option compared with topical anesthetics because use of the latter may increase the severity of corneal damage (Ubels et al., 1982). Dilution with water or milk could be helpful in oral exposures. For eye injury, washing with a large amount of water could be supportive, while for necrotic skin lesions, surgical intervention may be essential. Recovery depends on the extent of injury and could take several months (Patocka and Kamil, 2011). Reports indicate that mast cell activation and histamine release could be involved in CX-induced inflammation, toxicity, and urticaria (Hennino et al., 2006; Jain, 2014; Tewari-Singh et al., 2017). Employment of therapies that can ameliorate anaphylactic symptoms and counteract mast cell activation-related release of inflammatory mediators like histamine alone or in combination is a pioneering strategy for investigation to ameliorate CX-induced morbidity and mortality from its cutaneous exposure. Additionally, second-generation antihistamine with mast cell-stabilizing properties, analgesics, and antibiotics could be given to reduce pain, prevent infections, and promote healing.

13.6 Concluding remarks and future directions

CX is a manufactured urticant or nettle agent, and is a highly reactive and corrosive chemical warfare agent. Although it has never been used in warfare, its potent nature, fast penetration ability, easy synthesis, and toxic consequences make it a potential military and/or terrorist weapon. It could be produced easily and weaponized with other chemical warfare agents to increase their deleterious effects. Compared to other vesicants, CX is absorbed very rapidly through clothing and causes instantaneous and severe damaging effects to the skin, eyes, and mucus membranes with instant pain. Even though it is considered the most potent and damaging vesicant that can be used for rapid incapacitation and death, very little is known regarding the toxic effects and mechanism of action of CX following its exposure, and no antidote is available. To overcome these limitations, comprehensive studies to investigate the pathophysiology of the toxic effects of CX are needed to develop effective targeted therapies.

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Chapter 14

Psychotomimetic agent BZ (3-quinuclidinyl benzilate)

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14.1 Introduction

Experiences with the use of chemical warfare agents (CWAs) during World War I and under the pressure of general public opinion, have led to the search for novel chemicals with decreased lethal effects for military use (Ketchum et al., 1973). This research was focused on contemporary incapacitants influencing behavior with no severe/irreversible effects on important vital functions. An incapacitant is a chemical agent which causes a disabling condition that persists for hours to days after exposure to the agent. D-Lysergic acid diethylamide (LSD-25) was the first candidate for such an incapacitating agent. LSD-25 causes excessive neuronal activity by facilitating neurotransmission. However, its physical-chemical properties and unpredictable postexposure behavior led to the exclusion of LSD-25 from military research.

Other incapacitating agents included the esters of glycolic acid, that is, atropine-like anticholinergic compounds. The psychotomimetic effect of some natural anticholinergic substances has been known since ancient times. The first mention of deadly nightshade (*Atropa belladonna*) and its effect comes from a Greek philosopher and naturalist, Theophrastus (331−287 BCE), known as the "father of botany." From ancient times to the Middle Ages, atropine and other natural alkaloids were commonly used for religious ceremonies, Sabbaths, or as a tool of poisoners. The beginnings of medical use for these substances date back to the 17th century. In the 1950s, various anticholinergic drugs were investigated for potential military and industrial uses, including agent BZ. BZ (also known as *3-quinuclidinyl benzilate*, *QB*, *QNB*, *BZ agent*, *Buzz agent*, *Ro 2-3308*, or *EA 2277*) is a prototype of the CNS depressants; a prototype of CNS stimulants are LSD-25, cannabinol, or amphetamines.

Among the many tested compounds, anticholinergic drugs were chosen as promising for further research. The anticholinergics are generally "glycolates" (substituted glycolic and tropic acid esters) of which the representative and best-known member is atropine. Major symptoms of low-level atropinization include xerostomia ("dry mouth"), mydriasis, and tachycardia. Under high doses of atropine, central excitation becomes more prominent, leading to restlessness, irritability, disorientation, hallucinations, or delirium. Excessive doses caused depression, coma, and medullary paralysis.

A number of anticholinergic compounds influencing higher nervous functions have been tested (Table 14.1).

This group of compounds appears to have both peripheral and central properties. The psychotomimetic effects of atropine have been known since antiquity (the use of *Atropa belladona* extracts to induce hallucinations during Sabbaths and other rituals). The central effects of cholinergic compounds increase in the following order: atropine, scopolamine, benactyzine, ditrane and, finally, BZ and other esters of glycolic acid (Albanus, 1970). BZ was originally investigated as an antispasmodic drug in the therapy of gastrointestinal diseases. Nevertheless, even low excesses in dosing were likely to cause severe side effects including confusion and hallucinations. Therefore, BZ was withdrawn from commercial studies and referred to the US military forces as a possible candidate as an incapacitating agent (Sidell et al., 1997). Nowadays, BZ is used in research into cholinergic neurons in the peripheral nervous system (PNS) and CNS, nonneuromuscular cells and tissues for identification of muscarinic receptors

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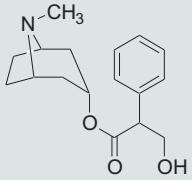
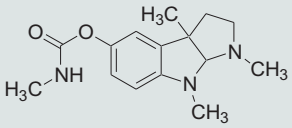
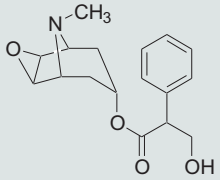
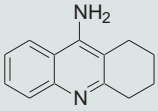
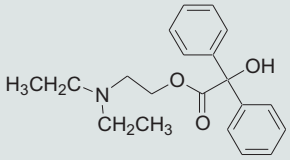
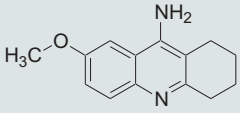
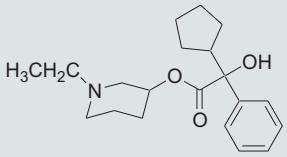
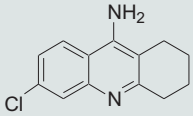
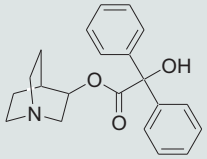
TABLE 14.1 Structural formulae of selected anticholinergic psychotomimetics and antidotes (cholinesterase inhibitors).

Anticholinergics		Antidotes	
Atropine		Physostigmine	
Scopolamine		Tacrine	
Benactyzine		7-Methoxy tacrine	
Ditrane		6-Chlorotacrine	
BZ, 3-quinuclidinyl benzilate			

via several laboratory techniques, mainly radioligand binding assays (Monica et al., 2008; Yamada et al., 2008; Soukup et al., 2011). BZ has also been implicated in several experimental epilepsy models (Schneider and de Lores Arnaiz, 2013), animal models in Parkinson's disease (Knol et al., 2014), animal behavioral studies (Misik et al., 2014), neurodegenerative disorders, such as Alzheimer's disease (AD), and other types of dementia (Wyper et al., 1993; Pakrasi et al., 2007; Schliebs and Arendt, 2011). In the 1960s, many similar compounds including BZ were studied in the framework of the military through industrial liaison programs (Pearson, 2006); following the criteria of politico-military goals and requirements, the general characteristics of an optimal incapacitating agent were:

- Low effective doses (acting in μg/kg or less);
- Rapid onset (minutes);
- Defined duration (optimally minutes to hours)
- Reversibility with no permanent effect;
- Stability at storage and delivery;
- Significant and predictable effect;
- Capability of rapid dissemination in defined conditions;
- High safety ratio.

14.2 Background

BZ is a white crystalline substance with a bitter taste that is soluble in aqueous and organic solvents. It is stable at field conditions for at least 1 or 2 days with no loss of its incapacitating activity. BZ is effective by all routes of administration and is metabolized primarily in the liver and excreted by the kidneys. After exposure to an effective dose (ED), mild peripheral effects occur within 1 h and central effects occur after about 4 h. The central effects peak at 8–10 h and last 24–48 h. The physicochemical properties are summarized in Table 14.2.

TABLE 14.2 Physicochemical properties of 3-quinuclidinyl benzilate (BZ, QNB).

Appearance	White crystals	References
Molecule weight	337.39	
Melting point	190°C (racemic 168°C)	Aleksandrov and Emel'yanov (1990)
Boiling point	412°C	Aleksandrov and Emel'yanov (1990)
Volatility	c_{max} = 0.0005 mg/L (by 70°C)	Aleksandrov and Emel'yanov (1990)
Solubility of hydrochloride	Very good	
Stability in substance	Compound is stable	
Stability in solution	Compound is stable in the water solution	
Persistence in terrain	Air oxidation proof (half-time 3–4 weeks) by 25°C and pH 7	Aleksandrov and Emel'yanov (1990)
Method of determination in field conditions	In Czech army: 1. Indicative tube type PT-51, (producer Oritest Ltd., Prague) 2. In field laboratory PPCHL-90	
Method of determination in biological agents under laboratory conditions	Determination: extract spectrophotometry of ion pairs	Halámek and Kobliha (1993)
	Detection: TLC	Skalican et al. (1997)
	HPLC/UV detection or LC-MS	Herman et al. (2019)

14.3 Toxicokinetics and mechanism of action

BZ is absorbed by all usual routes of administration (oral, parenteral, and inhalation). When administered by inhalation, the absorption into the transport system (bloodstream) is more pronounced in comparison with oral administration. Experimental studies were performed with parenteral administration and, in this case, i.v. administration is without losses because of direct involvement of BZ in the transport system. The compound binds to the plasma proteins (preferably albumin) and is transported to the target point of the toxic effect—the CNS and PNS. At these localizations, BZ interferes with cholinergic nerve transmission at muscarinic sites, both in the peripheral autonomic nervous system and in the brain and spinal cord. Due to the wide distribution of muscarinic receptors, effects are observed upon almost every phase of neural regulation. BZ readily crosses the blood–brain barrier (BBB) and is distributed to all areas of the brain and spinal cord where it interacts with cholinergic receptors as a competitor of the physiologically active neurotransmitter ACh. The effect is manifested as a relative lack of acetylcholine. At the periphery, BZ binds to acetylcholine muscarinic receptors (e.g., of smooth muscles), similarly to atropine, with a strong antagonistic effect on receptors (Fusek et al., 1971; Table 14.3).

TABLE 14.3 Pharmacological activity of compounds tested on isolated rat jejunum.

Compound	i.a.	$pD_2 \pm P_{95}$	$pA_2 \pm P_{95}$
Acetylcholine	$\alpha = 1$	6.79 ± 0.06	
Muscarine	$\alpha = 1$	6.59 ± 0.05	
Atropine	$ß = 0$		8.92 ± 0.05
Benactyzine	$ß = 0$		7.64 ± 0.07
Scopolamine	$ß = 0$		8.83 ± 0.06
Ditrane	$ß = 0$		8.36 ± 0.07
BZ	$ß = 0$		8.55 ± 0.09

α, Intrinsic activity of agonist; $ß$, intrinsic activity of antagonist; *i.a.*, intrinsic activity; P_{95}, 95% confidence limits; pA_2, negative decadic logarithm of antagonist ED_{50} (−log ED_{50} antagonist); pD_2, negative decadic logarithm of agonist ED_{50} (−log ED_{50} agonist).

At the ACh, BZ binds to all subtypes of muscarinic receptors (M1–M5), each showing different functions in the brain (Lefkowitz, 2004). The particularly long duration of the central action of BZ may be related to its higher affinity for nervous tissue, especially the strong adsorption by mitochondria. This feature of BZ leads to a reduction in oxygen consumption by nerve cells, which is stimulated in various ways (Jovic and Zupanc, 1973).

14.4 Toxicity

The acute toxicity, expressed as LD_{50}, for various species and routes of administration is shown in Table 14.4.

In general, the acute toxicity of anticholinergic drugs is relatively low and the dose causing incapacitation is substantially lower. Therefore the ratio between the lethal and incapacitant doses is in the range of a logarithmic scale.

The effective doses (EDs) of some psychotomimetic drugs for humans are shown in Table 14.5.

BZ at a single dose of less than 1 mg produces delirium lasting several days. The safety margin (ratio of lethal to incapacitating dose) for man is estimated to be at least 30. The median effective dose (ED_{50}) of BZ under field conditions is approximately 60 mg/min.m^3 for a man of 75 kg body weight with a volume of respiration of

TABLE 14.4 Lethal doses of 3-quinuclidinyl benzilate, atropine, and scopolamine.

Species	Route of administration	LD_{50} (mg/kg)		
		3-Quinuclidinyl benzilate (BZ)	Atropine	Scopolamine
Mouse	i.v.	22	74	163
	i.p.	110	256	119
	i.m.	42		
	p.o.	460	693	
Rat	i.v.	20	41	
	i.p.		256	
	i.m.	281		
	p.o.		733	1270
Guinea pig	i.v.	14	163	
	i.p.		277	
	p.o.		1100	
Rabbit	i.v.	10		
	i.m.		588	
Cat	i.v.	12		
Dog	i.v.	12		
Pig	i.v.	5		

TABLE 14.5 The effective doses (ED) of some psychotomimetic drugs for incapacitation in man.

Drug	Route of administration	ED (mg/kg)	References
LSD-25	i.m.	0.0005–0.001	Hofmann (1960)
	p.o.	0.001	Hollister (1968)
BZ	i.m.	0.006	Sidell et al. (1973)
	i.m.	0.01	Spivak and Milstein (1973)
	i.v.	0.005	Spivak and Milstein (1973)
Scopolamine	i.m.	0.024	Ketchum et al. (1973)
Atropine	i.m.	0.175	Ketchum et al. (1973)
Ditrane	i.m.	0.1-0.3	Gershon and Olariu (1960)
	i.m.	0.15	Ketchum et al. (1973)

15 L/min. Rodents seem to be generally more resistant to the effects of BZ. Doses between 2.0 and 10.0 mg/kg administered intramuscularly induced only mild to moderate signs of anticholinergic stimulation (e.g., hyperactivity, piloerection, mild tremor) which diminished spontaneously within approximately 1 h (Misik et al., 2014).

14.5 Symptoms

BZ is effective via intravenous, intramuscular, respiratory, or oral routes of administration. The effect of BZ in a case of percutaneous administration, when mixed with suitable solvent, is limited. The limited data available indicate that the higher doses caused stronger effects and prolonged duration. In 1960–69, field tests of BZ were conducted by the US military. Sidell's (1982) description of the effects induced by BZ (4.5–17.1 μg/kg) is as follows.

At low doses, the effects include a dry mouth, decreased gastric motility, inhibition of sweating, an increase in heart rate, mydriasis and loss of accommodation, mild sedation, and mental slowing.

These effects intensify under higher doses. There are marked disturbances of cognitive and locomotor functions at all levels of CNS, including alteration of motor coordination and a decline in attentiveness and control of thought and learning process. Confusion, restlessness, impairment in perception, interpretation, and memory disturbances are observed. The first symptoms occur dependent on the route of administration; judgment and deficient insight are all features of this syndrome. True hallucinations are present. Under excessive doses, the subject may become stuporous or even comatose for several hours.

After a single intramuscular injection (BZ doses of 5.0–6.4 μg/kg) the following symptoms were observed:

- 10 min: lightheadedness and giggling;
- 30 min: xerostomia, blurred vision, nausea, chilly sensations, and twitching;
- 1 h: flushed skin, lack of coordination, fatigue, unsteadiness, sleepiness, and quivering legs;
- 2 h: many of the above symptoms, plus poor concentration, restlessness or sleepiness, hallucinations, slurred speech, and muscle fasciculation;
- 3 h: the above symptoms, plus tremors;
- 4 h: the above symptoms, plus difficulty in handling the subject and an increased pulse rate up to 130;
- 8 h: the above symptoms, plus delirium and hallucinations;
- 24 h: persistent delirium, hallucinations, restlessness, unsteadiness, and increased pulse rate in some (but not all) subjects;
- 48 h: persistent impairment of cognitive functions.

Mydriasis and disturbed accommodation remain unchanged depending on the dose at 2 or 3 days. The mean time of incapacitation is about 70 h. In real-life situations, wide variations in absorbed dose would occur, and thus the intensity and duration of symptoms will also vary over a large time scale.

14.6 Risk assessment

BZ was stockpiled by the US military forces in 1980. In 1992, the US delegation at the Conference on Disarmament in Geneva declared that all stocks of BZ had been destroyed. However, it cannot be excluded that BZ has been stored by some other armed forces (no official data available). The military use of BZ was limited to special operations and, at present, BZ agent can be considered as a potential agent for special military operations. Nowadays, the threat presented by a group of incapacitants (other than anticholinergic hallucinogens) known as "nonlethal chemicals" with extremely strong effect, is real (e.g., fentanyl derivatives involved in the Moscow theater incident, 2002). The potential misuse of similar compounds by terrorist groups cannot be excluded, as well as use against groups of people or aimed groups of people, for example, military staff (Pearson, 2006).

14.7 Treatment

The treatment of BZ intoxication is in principle based on reversion of the mechanism of action (simply described as a lack of neuromediator ACh). Generally, for an increase in the ACh level, inhibitors of cholinesterases are the drugs of choice. However, use of the majority of these compounds is limited due to small ratio between the therapeutic and toxic doses. Reversible inhibitors are preferred, although some experiments with highly toxic nerve agents (VX and sarin) as an antidote against BZ have been performed on healthy volunteers (Sidell et al., 1973). Peripherally acting anticholinesterase drugs that do not cross the BBB (e.g., pyridostigmine, neostigmine, and pilocarpine) are ineffective against the central effects of BZ.

Physostigmine was the first antidote against BZ. Its antidotal effect against *A. belladona* extract has been known for more than 150 years. It is known that physostigmine acts as a potent antidote against scopolamine intoxication and is able to mitigate all symptoms on electrophysiological, psychiatric, and biochemical levels. Physostigmine was considered as a potential antidote against anticholinergic incapacitants including esters of glycolic acid. Administration of physostigmine (2–3 mg i.m.) is required to alleviate symptoms of BZ intoxication. Repeated injections at intervals of approximately 15 min to 1 h may be required to build appropriate levels. Once a

desirable effect is achieved, it should be maintained by slow intravenous injection or infusion. Doses of 2−4 mg every 1−2 h may be required. Oral dosing (2−5 mg every 1−2 h) should replace i.v. therapy as soon as possible.

Nevertheless, the therapeutic index of physostigmine is rather narrow, showing side effects and short time duration of the therapeutic effect. Therefore attempts have been made to obtain new inhibitors with lowered toxicity in comparison with physostigmine. Acridine derivatives were of great interest (Albert, 1966). From these compounds, 1,2,3,4-tetrahydro-9-aminoacridine (Fusek et al., 1974), tacrine, was found to be effective not only against anticholinergics but also against morphine and curare overdosage. Tacrine is an inhibitor of cholinesterases comparable with neostigmine or physostigmine and also showing antihistaminic activity. Its effect is prolonged in comparison with physostigmine. Historical studies investigating antidotes against anticholinergic hallucinogens recognized tacrine as the most promising antidote at that time (Gershon and Angrist, 1973). The antidotal effect of tacrine against ditrane intoxication including improvement of the EEG misbalances was approved by Itil and Fink (1966) in 74 patients. Atropine, scopolamine, and ditrane intoxications were successfully treated using physostigmine and tacrine but also sarin (Ketchum et al., 1973). However, the side effects of tacrine, including hepatotoxicity, were the reason for its limited use and finally, it was abandoned (Marx, 1987).

7-Methoxy derivative of tacrine (7-MEOTA) has been synthesized and tested (Fusek et al., 1986). 7-MEOTA inhibited in vitro preferably butyrylcholinesterase (BuChE) ($I_{50} = 3.5.10^{-7}$ M) rather than acetylcholinesterase (AChE) ($I_{50} = 3.5.10^{-6}$ M). The inhibition is of a competitive−noncompetitive reversible type. The characteristics of AChE inhibition indicate that 7-MEOTA binds on the active surface of AChE in the gamma-anionic site, like galantamine or coumarin. Inhibition of blood AChE by *O*-ethyl-*S*-(2-dimethylaminoethyl) methylphosphonothioate (0.042 mg/kg = 1 × LD_{50}, i.m.) after premedication with 7-MEOTA (100 mg/kg, i.m.) was substantially lower than in intoxicated animals pretreated with saline, which underlines its possible use for preventing intoxication by organophosphorus anticholinesterase agents. 7-MEOTA enhanced the contraction response of the guinea pig atria (ED_{50} positive inotropic effect = 1.7×10^{-6} M), reduced the frequency of contractions (by binding the compound to the effectors of the cholinergic system of the heart tissue), increased the contraction response of the isolated rat diaphragm, and antagonized the effect of D-tubocurarine. The dose of 3×10^{-6} M of 7-MEOTA entirely suppressed the effect of a dose of 2×10^{-6} M D-tubocurarine. 7-MEOTA elicited a contraction response (1×10^{-7} M) and intensified the response of the isolated rat jejunum to the applied concentration of cholinomimetics. The long-term increase of intestinal peristaltic function after low concentrations of 7-MEOTA (1×10^{-7} M) testifies to the inhibitory effect of the compound on tissue cholinesterases (Fusek et al., 1979). On the other hand, the surface cutaneous electrogastrography did not prove a significant impact of 7-MEOTA on dominant frequency of gastric slow waves in pigs. In this study 7-MEOTA caused only a short-term late increase in the power ratio (ratio of the areas of amplitudes after and before study drug administration) at 60 min after administration. Blood cholinesterase activity did not correlate with any EGG parameter (Bureš et al., 2015). Depending on the dose, 7-MEOTA effectively antagonized symptoms of intoxication elicited by anticholinergics in dogs. A dose of 5 mg/kg of 7-MEOTA shortened the time needed for a fall in the score of intensity of symptoms elicited by BZ (0.05 mg/kg) from 210 min in untreated intoxication to 54 min, respectively (Fusek et al., 1979).

Besides the direct influence on effectors of the cholinergic system, the effect of 7-MEOTA might be mediated via the induction of cholinesterase inhibition in the CNS and PNS. Additional beneficial properties of 7-MEOTA in comparison with physostigmine suggest the possible therapeutic use of this compound in cases where hitherto physostigmine was used before. In contrast to physostigmine, there is no need for repeated doses to maintain the therapeutic effect of 7-MEOTA at low dosage and, moreover, minimal side effects appear.

This medicament has not only use as an antidote against BZ intoxication but it can be used in general as a drug for the treatment of cholinergic disturbances such as AD. 7-MEOTA is a potent, centrally active cholinesterase inhibitor. In isolated rat jejunum, 7-MEOTA increased muscle contractility and in isolated guinea pig ventricular myocytes this compound was found to prolong transmembrane action potential and decrease amplitude of the plateau. In isolated rat diaphragm, 7-MEOTA increased muscle contractility after electric stimulation of the phrenic nerve.

7-MEOTA was found to antagonize the convulsive action of pentamethylenetetrazole and significantly decreased the number of surviving animals following administration of this drug. Results from behavioral studies indicated that 7-MEOTA antagonized anticholinergic syndrome evoked by scopolamine, ditrane, and BZ. Nevertheless, some contemporary anticholinergic drugs used for therapy of neurodegenerative disorders including rivastigmine or donepezil and some novel experimental compounds derived from 7-MEOTA showed increased effectiveness against 7-MEOTA in laboratory rats (Misik et al., 2015).

The antidotal effect of 7-MEOTA on the anticholinergic syndrome was potentiated by nootropics, diazepam, and especially opioid peptides (Fusek, 1977).

TABLE 14.6 Lethal doses of tacrine, 7-methoxytacrine, physostigmine, and 6-chlorotacrine in laboratory animals.

Compound	Animal	Route of administration	LD_{50} (mg/kg)
Tacrine	Mouse	i.m.	28.9 (24.2–35.8)
	Rat	i.m.	33.8 (28.8–40.6)
	Rat	p.o.	103.7 (84.6–135.8)
	Rat	i.p.	20.2 (16.4–24.8)
	Rat	i.v.	12.0 (10.7–13.6)
	Rabbit	i.n.	13.3 (8.0–18.8)
	Dog	i.m.	12.6 (10.1–14.7)
7-Methoxytacrine	Mouse	i.m.	125 (110–143)
	Rat	i.m.	258 (224–313)
	Rat	p.o.	793 (662–950)
	Rat	i.p.	73.3 (61–90)
	Rat	i.v.	22.2 (19.9–25.2)
	Rabbit	i.m.	75.3 (60–89)
	Dog	i.m.	18.9 (15.2–22.1)
Physostigmine	Mouse	i.m.	0.86 (0.7–1.0)
	Rat	i.m.	2.2 (1.9–2.4)
	Dog	i.m.	0.83 (0.68–0.95)
6-Chlorotacrine	Rat	i.p.	9.0 (6.8–12.3)

7-MEOTA also antagonized the side effects of tricyclic antidepressants and protected AChE against inhibition by some organophosphate anticholinesterase compounds.

As is shown in Table 14.6, the acute toxicity of 7-MEOTA was rather low; LD_{50} (i.m.) was 125 mg/kg for mice and 258 mg/kg for rat. With p.o. administration to rat LD_{50} was 793 mg/kg. Analogous values for tacrine at the same order were: 29, 34, and 104 mg/kg (Fusek, 1977). 7-MEOTA was found to be markedly less toxic than tacrine also for dogs and rabbits. Subacute toxicity study of 7-MEOTA (3-months administration) demonstrated that the compound was well tolerated at doses of 25 mg/kg (i.m.) and 50 mg/kg (p.o.) in rats and at a dose of 2.5 mg/kg (i.m. and i.v.) in Beagle dogs. No pathological changes were observed in biochemical, hematological, and morphological investigations.

Some recent studies investigated the antidotal effect of several novel analogs of tacrine, donepezil, and 7-MEOTA (Misik et al., 2015, 2016, 2018). One of numerous derivatives is 6-chlorotacrine hydrochloride (6-Cl-THA; 6-chloro-9-amino-1,2,3,4-tetrahydroacridine hydrochloride, Table 14.1) containing a chlorine atom in the C6 position, which strongly increases inhibitory potential compared to the parent compound. Accordingly, a significantly increased inhibitory effect against the parent compound was observed in vitro and some other advantages, such as enhanced blood–brain barrier penetration and increased selectivity toward AChE were proposed. Thus 6-Cl-THA was utilized in the development of novel dual binding site inhibitors or multitarget-directed ligands (Camps et al., 2009; Di Pietro et al., 2014; Eckroat et al., 2013; Nepovimova et al., 2014; Liao et al., 2015; Nepovimova et al., 2015). In vivo evaluation of 6-Cl-THA revealed a procognitive effect in several behavioral tasks. Rats administered with 1.8 mg/kg of 6-Cl-THA showed improved spatial navigation in a water maze, even resembling the performance of untreated animals (Misik et al., 2018). Unfortunately, chlorine increased toxicity of 6-Cl-THA in relation to the parent compound and the LD_{50} of 6-Cl-THA in rats is three times lower compared with parent compound tacrine and eight times lower compared with 7-MEOTA. Thus, increased toxicity of 6-Cl-THA is the main limiting factor. Other modification of the molecule or hybridization leading to decreased toxicity and increased biological efficacy might be the solution.

14.8 Analytical methods

BZ determination in biological samples and other complex matrices is possible using HPLC analysis with UV detection or LC-MS/MS technique. The sensitive and rapid LC-MS/MS method for agent BZ determination in plasma and tissue homogenates was developed by Herman et al. (2019) for the purpose of investigating its fate in the organism. Sample preparation was based on solid-phased extraction on C-18 cartridges and the chromatographic separation was performed on a modified C-18 stationary phase with alkaline (pH 11) mobile phase which reflects the alkaline character of the target compound. Mass spectrometry was accomplished on a linear ion trap instrument, coupled with a heated electrospray ionization probe operated in the positive ion mode. The assay was linear in the concentration range 0.5−1000 ng/mL ($r^2 = 0.9947$) with the limit of detection (LOD) 0.15 ng/mL, and the lower limit of quantification (LLOQ) 0.5 ng/mL. Described parameters were found to be sufficient to measure levels of BZ in rat plasma and tissue samples. The maximum concentration of BZ in plasma and brain tissue was measured 3 and 5 min after intramuscular administration, respectively (Herman et al., 2019). The gass chromatography represents another approach to the BZ determination in biomatrices. A method of the BZ and its metabolites assessment in urine based on gass chromatography coupled with mass spectrometry was published for example by Byrd et al., 1992.

At present, the BZ compound is frequently used as a radiolabeled ligand of muscarinic receptors in order to measure anticholinergic activity (Schreiber et al., 2018) using a radioreceptor assay developed by Tune and Coyle (1980) or binding capacity of muscarinic receptors using the time course of radioactivity concentration assay (Inoue et al., 2018).

The fluorometric method for 7-MEOTA determination in biological material was developed and blood-concentration profiles of 7-MEOTA in rats and healthy volunteers were estimated (Filip et al., 1991).

Maximal concentrations of 7-MEOTA in human blood were observed approximately 0.5−1 h after i.m. and 4 h after p.o. application of drug. The half-life was 5 h, and the effective level was maintained for 12 h. Similar results in rats with radiolabeled (H^3) 7-MEOTA were obtained (Patocka et al., 1996).

In healthy volunteers, 7-MEOTA was well tolerated in q single dose of 2 mg/kg (p.o.) or 1 mg/kg (i.m.) following daily administration (7 days). The compound did not influence the cognitive functions of healthy persons. The blood-concentration profiles of volunteers corresponded well with those in rats. Clinical testing of this drug was performed on patients with tardive dyskinesias which occurred after long-term administration of neuroleptics with good therapeutic efficacy (Zapletalek et al., 1989).

Based on the results obtained, tablets of 7-MEOTA (100 mg) and injections (50 mg in 2 mL) were introduced into the Czech armed forces as an antidote against psychotomimetic agents. Modern analytical instrumentation also enables determination of 7-MEOTA in biological samples. Soukup et al. (2013) developed an HPLC method based on reverse phase separation and fluorescent detection (excitation and emission wavelength 240 and 360 nm). The LOD and LLOQ in plasma samples were 2.10 and 6.25 ng/mL, respectively.

14.9 Agent BZ in behavioral research

Proper functioning of the central cholinergic system is crucial for cognition. A shortage of cholinergic transmission in target areas of the brain (prefrontal cortex, hippocampus, striatum, or amygdala) is connected with behavioral alterations including impairments of learning and memory. Thus, some compounds with anticholinergic activity, such as scopolamine, atropine, trihexyphenidyl, biperiden, or pirenzepine have been investigated for their behavioral and neurochemical effects (Bymaster et al., 1993; De-Mello and Carobrez, 2002; von Linstow Roloff et al., 2007; Klinkenberg and Blokland, 2010; Doguc et al., 2012) aimed at finding an appropriate experimental model of neurodegenerative disorders in laboratory animals.

As a standard drug, mainly scopolamine was used. Nevertheless, agent BZ has been several times investigated and proved to be a potent compound able to simulate cognitive impairment in experimental animals, similar to those observed in patients with Alzheimer's disease.

The effect of BZ seems to be very similar to the effect of scopolamine with the difference of prolonged action on behalf of BZ. The amnesic effect of 2.0 mg/kg of BZ in Wistar rats was observed up to 24−48 h postadministration (Misik et al., 2014). In contrast, the effect of scopolamine is usually shorter, up to approximately 8 h (Fig. 14.1).

The effect of agent BZ is specifically modified across the stages of learning and memory processes, including acquisition, consolidation, and retrieval. However, the influence on acquisition is extensive, and minimal affection of retrieval and consolidation was observed (Misik et al., 2014; Fig. 14.2). Experimental animals administered with BZ before training (acquisition stage) were less effective in behavioral tasks, whereas a minor effect was observed when the agent was administered to trained animals exposed to a retrieval behavioral task (Fig. 14.2). This finding corresponds to the hypothesis that the cholinergic system is mainly essential for acquisition, and the

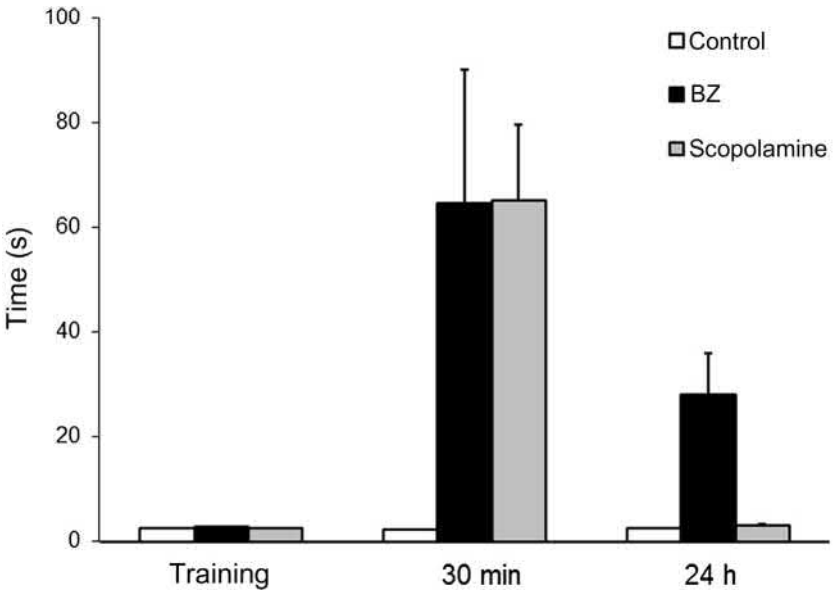


FIGURE 14.1 The prolongation of passage times in male Wistar rats performing the multiple T-maze test 30 min and 24 h postadministration of agent BZ (2.0 mg/kg) and scopolamine (0.3 mg/kg). The multiple T-maze task is focused on spatial navigation.

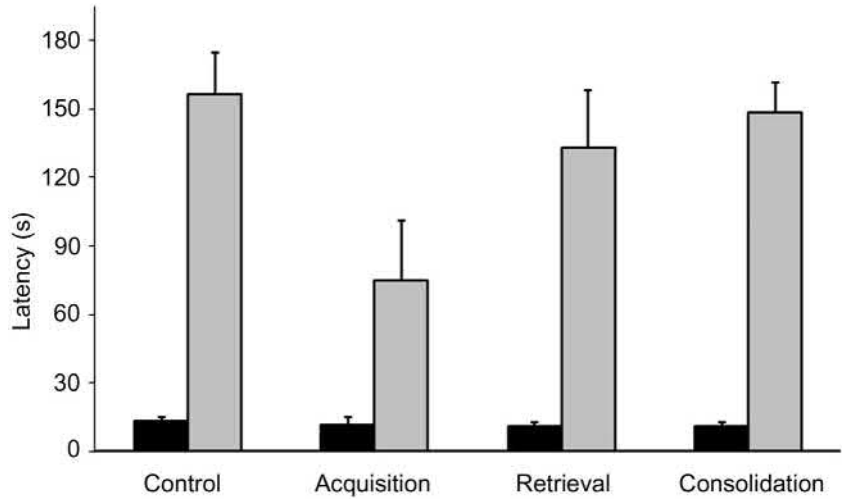


FIGURE 14.2 Effect of agent BZ on acquisition, retrieval, and consolidation in male Wistar rats performing passive avoidance task. There is training (*black columns*) and test (*gray columns*) entrance latency of control versus BZ-treated rats (2.0 mg/kg). Agent BZ was administered according to three different protocols aimed to affect the specific stage of learning and memory process. The passive avoidance task is based on fear conditioning.

blockade of M receptors during the acquisition stage impairs memory formation (Deiana et al., 2011).

In general, BZ may serve as an effective, long-acting alternative to the commonly used scopolamine in animal models of cognitive deficits, whereby new drugs (mainly cholinesterase inhibitors) could be tested.

14.10 Concluding remarks and future directions

BZ was recognized as an incapacitant agent for military use. It was stockpiled and stored as a CWA by the US military, but all stocks were destroyed in 1992. Nevertheless, the use of this agent is not completely excluded. Its mechanism of action is based on the interaction with cholinergic receptors. The symptomatology is characterized by peripheral action (vegetative symptoms) and the number of central symptoms affecting cognition and behavior. The effective antidotal therapy is of vital interest and it is based on the main principle of cholinesterase inhibition in the CNS, possibly using commonly available physostigmine or 7-MEOTA, eventually donepezil and rivastigmine, according to current animal data. Finally, the use of BZ in research into the cholinergic nervous system cannot be omitted.

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Chapter 15

Fluoroacetate

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15.1 Introduction

Fluoroorganic compounds attracted the attention of researchers more than 70 years ago when among a large class of biologically inert chemicals a group of very toxic compounds was revealed, having the general formula CH_2FCOOR and the common name "fluoroacetate" (FA). The toxicological effects of FA do not become apparent immediately, even after exposure to lethal doses, but only after a latent period of half an hour to several hours for animals and humans. The level of FA in some plants can reach up to 5 g/kg dry weight (Hall, 1972) and it can cause death of livestock and domestic animals, sometimes with appreciable economic damage (McCosker, 1989; Minnaar et al., 2000a; Lee et al., 2012). FA can be found in fog and raindrops in some industrial regions (Rompp et al., 2001). The best known representative of FA is its sodium salt (SFA, compound 1080). This substance is used in several countries for controlling populations of some vertebrates, and sometimes causes the death of pasture animals (Giannitti et al., 2013). There are also series of fluorocompounds whose metabolism is connected with the formation of FA, these are antineoplastic drugs (5-fluorouracil and isomers of fluoronitrosourea); *N*-(2-fluoroethyl) derivatives of the narcotic analgesics normeperidin and normethazocin; pesticides, 1,3-difluoro-propanol and fluoroacetamide (FAA, compound 1081); and 1-(di) halo-2-fluoroethans and fluoroethanol (Reifenrath et al., 1980; Tisdale and Brennan, 1985; Feldwick et al., 1998). The urgency of the problems associated with FA toxicity and the need for an effective therapy for acute intoxication has greatly increased in connection with a new threat—international terrorism (Holstege et al., 2007). The physicochemical features of FA, the lack of taste and odor, delayed manifestation of toxicity, and the similarity of clinical signs of intoxication to some natural indispositions are these characteristics that necessitate comprehensive studies of the mechanisms of action of FA and the search for effective therapeutic means for treatment of acute intoxication.

15.2 Background

FA was initially synthesized in 1896 and decades after that was found in *Dichapetalum*, *Gastrolobium*, *Oxylobium*, *Acacia, Palicourea, Mascagnia, Tanaecium*, and *Amorimia* plants growing in Australia, South and Central Africa, and South America (Oerlichs and McEwan, 1961; de Oliveira, 1963; McEwan, 1964; Aplin, 1971; Vickery et al., 1973; Camboim et al., 2012a; Lee et al., 2012; Esters et al., 2013). Chemically pure FA is a very stable compound, and the energy of dissociation of the fluoro−carbon bond in the molecule is regarded as one of the highest among the natural compounds (Ichiyama et al., 2004). However, FA is broken down in biological preparations from plants (Minnaar et al., 2000a). In soils, the major degradation pathway for SFA is through microbial degradation to the hydroxyl metabolite, hydroxyacetic acid, and microbial mineralization to CO_2; temperature, rather than soil type or moisture content, is the dominant factor affecting the rate of degradation (Northcott et al., 2014). Seven bacteria from soil and plant samples able to degrade SFA were identified by 16S rRNA gene sequencing as *Paenibacillus* sp. (ECPB01), *Burkholderia* sp. (ECPB02), *Cupriavidus* sp. (ECPB03), *Staphylococcus* sp. (ECPB04), *Ancylobacter* sp. (ECPB05), *Ralstonia* sp. (ECPB06), and *Stenotrophomonas* sp. (ECPB07) (Camboim et al., 2012a). Also, two SFA-degrading bacteria from

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caprine rumen, *Pigmentiphaga kullae* (ECPB08) and *Ancylobacter dichloromethanicus* (ECPB09) (Camboim et al., 2012b), and one from bovine rumen belonging to the phylum Synergistetes (Davis et al., 2012) have been identified, the last functioning in anaerobic conditions.

After inhalation or ingestion, FA is easily absorbed by tissues and its high toxicity is independent of its route of entry into organisms (Chenoweth, 1949). The mechanism of toxic action of FA is widely known as "lethal synthesis" (Peters, 1952; Peters and Wakelin, 1953), the essence of which is conversion of nontoxic FA to toxic fluorocitrate (FC) within the cells of an organism. The main causes of death are considered to be imbalance of intracellular ions, osmotic imbalance, and a deficit of ATP as a consequence of aconitase blockade (Buffa et al., 1973). The latent period, from the moment of poisoning with FA to the manifestation of clinical signs, is 0.5−3 h (in warm-blooded animals). This period reflects penetration of FA into blood and cells and conversion of FA to FC, with the consequent uncoupling of intracellular metabolism. Death usually occurs within 24−48 h, but can be later. At autopsy there are no specific signs of intoxication (Peters et al., 1981). For those warm-blooded animals least adapted to FA the lethal dose is less than 2 mg/kg (Atzert, 1971). However, there is a considerable species-specific difference in clinical signs of intoxication and differences in sensitivity to the poison (Chenoweth, 1949). The mean lethal dose varies within the range from 0.05 mg/kg for dogs to 150 mg/kg for possums. The most common criterion of tolerance, or vice versa sensitivity, of animals to FA is intensity of metabolism. Thus, in the lizard *Tiliqua rugosa* the level of metabolism of FA is 10-fold lower in comparison with that of the rat *Rattus norvegicus*, and the lethal dose for the lizard is 100-fold higher than that of the rat (Twigg et al., 1986). A low intensity of metabolism means a low conversion of FA to FC, which makes possible more effective excretion and detoxification. In the absence of specific clinical, physiological, and morphological signs of intoxication, determination of FA in tissues together with citrate and fluoride ions can be a diagnostic confirmation of FA intoxication (Schultz et al., 1982; Koryagina et al., 2006).

15.3 Toxicokinetics

15.3.1 Detoxification

The main pathway of detoxification of FA is its defluorination via a glutathione-dependent mechanism involving nucleophilic attack on the β-carbon atom and formation of fluoride and *S*-(carboxymethyl)glutathion, with subsequent cleavage of the latter into amino acids and an *S*-(carboxymethyl) conjugate complex excreted in the urine (Mead et al., 1979; Tecle and Casida, 1989). The highest defluorinating activity was found in the liver, followed by the kidneys, lungs, heart, and testicles in descending order. No defluorinating activity was found in the brain. The activity of enzymes responsible for defluorination depends on the glutathione (GHS) concentration with a maximum above 5 mmol/L, the apparent Km being 7 mmol/L at saturating concentrations of GHS (Soiefer and Kostyniak, 1983). Defluorination is mainly carried out by anionic proteins having glutathione transferase activity, though the anionic fraction contains nearly 10% of proteins without this activity but also capable of defluorination of FA. Moreover, cationic enzymes were shown to be responsible for about 20% of cytosolic defluorination of FA (Wang et al., 1986). The GHS-dependent enzyme defluorinating FA is not identical to GHS-dependent S-transferases; it is an FA-specific defluorinase with an acidic isoelectric point (pH = 6.4) and a molecular weight of 41 kDa (27 kDa for the main subunit) (Soiefer and Kostyniak, 1984). The activity and specificity of defluorinase isoenzymes vary markedly and are subjects of research presently (Tu et al., 2006; Nakayama et al., 2012).

15.3.2 Analytical procedure

The analysis of biological samples for FA is rather problematic because of the high polarity of the fluorine−carbon bond in the molecule. Liquid chromatography (LC) has been applied for analysis of FA in different media (Allender, 1990), and analysis of FA in plants and gastric contents by HPLC with UV detection has also been described (Minnaar et al., 2000b). Being a nonvolatile substance, FA was commonly analyzed by gas chromatography (GC), as a methyl derivative (Stevens et al., 1976), ethyl or *n*-propyl derivatives (Peterson, 1975), and as pentafluorobenzyl esters (Vartiainen and Kauranen, 1984). Derivatization with 2,4-dichloroaniline in the presence of *N*,*N*-dicyclohexylcarbodiimide was used for GC analysis of SFA in water (Ozawa and Tsukioka, 1987) and blood serum (Demarchi et al., 2001). A modified procedure by Eason et al. (1994) achieved low detection limits for FA at the level of 0.01 μg/g in plasma and urine, and 0.002 μg/g in tissue and feces of sheep and goats. However, this procedure is labor- and time-consuming, and the GC-electron capture detection procedure applied is considered to be unreliable at this level of sensitivity.

The main problem for GC analysis of FA in biological samples is co-elution of the matrix components. This can be overcome by sampling of the analyte from an equilibrium vapor phase. Static head-space analysis of SFA as ethyl fluoroacetate, with a linear range for SFA in water of 5−200 μg/mL and a detection limit of 0.5 μg/mL has been reported (Mori et al, 1996). Solid-phase microextraction (SPME) from an equilibrium vapor phase has

all the advantages of head-space analysis, whilst being a much more sensitive technique. We reported on a novel procedure for determination of FA in water and biological samples, involving ethylation of FA with ethanol in the presence of sulfuric acid, SPME of the ethyl fluoroacetate formed with subsequent analysis by GC-MS (Koryagina et al., 2006). To overcome the problem of the presence of the components co-eluting with FA derivatives we made use of GC-MS in the SIM (selective ion monitoring) mode. To avoid partial overlapping of the internal standard's peak with the sample matrices' components, quantification was performed with the use of two internal standards—carbon tetrachloride and toluene. The calibration plots for the determination of SFA in biological samples was linear in the SFA concentration range 0.01−5.0 μg/mL for both internal standards, and a linear relationship in blood plasma was observed in the range of 0.01−5.0 μg/mL ($r = 0.95$). With toluene as internal standard, the linear regression equation was $Y = 0.014\ X$ [Y was the ratio S(EthylFA)/S(toluene); X was the concentration of SFA, μg/mL]. The RSD (relative standard deviation) for fluoroacetate quantification at 0.1 μg/mL was 12% ($n = 5$). With carbon tetrachloride as an internal standard, a linear relationship in plasma was observed in the range of 0.01−5.0 μg/mL ($r = 0.98$). The linear regression equation was $Y = 0.1656\ X$ [Y was the ratio S(EthylFA)/S(CCl4); X was the concentration of FA, μg/mL]. The RSD for FA quantification at 0.1 μg/mL was 6% ($n = 5$), and the detection limit was 0.005 μg/mL ($S/N = 3$). The calibration characteristics of rat organ homogenates were identical to those of plasma. A decade has passed since our publication, but it seems that no sensible progress in analytical methodology has been achieved, taking into regard the levels of detection and quantification of FA in biological tissues (Liu et al., 2018).

15.3.3 Distribution in tissues and elimination

The data on toxicokinetics of FA are rather contradictory, apparently depending on analytical procedures and the dose of the poison; also, there is evidence for animal species specificity. The first data on toxicokinetics of FA demonstrated its rather uniform distribution between organs, with some predominance in the heart, brain, and kidneys (Gal et al., 1961). The half-life was calculated to be not less than 2 days, and this could cause secondary toxicity arising from ingestion of meat from poisoned animals (Aulerich et al., 1987). The half-life of FA was shown to be 10.8 h for sheep and 5.4 h for goats, that were administered 0.1 mg/kg; maximal concentration of FA 2.5 h after the poisoning was revealed in blood plasma (0.098 μg/mL), followed by kidneys (0.057 μg/g), skeletal muscles (0.042 μg/g), and liver (0.021 μg/g). Only traces of FA were found in all the tissues examined 96 h after

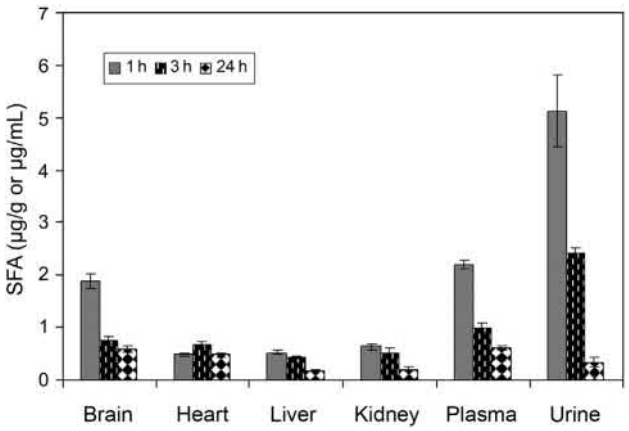

FIGURE 15.1 Data on the determination of FA (recounted as SFA) in rat organ homogenates and body fluids, at times following poisoning with SFA at a peroral dose of 2 mg/kg ($1/2LD_{50}$). Standard deviations (shown) were based on four to six replicate analyses.

the poisoning (Eason et al., 1994). At 1 and 12 h after introduction of SFA (0.25 mg/kg) to rats a similar ratio of FA was found in rat plasma (0.26 and 0.076 μg/mL, correspondingly) (Eason and Turck, 2002), the half-life period being 2.9 h. On the other hand, for rabbits under subacute intoxication with FA the half-life was found to be 1.1 h, and the level of FA in rabbit muscles, kidneys, and liver was much higher than in blood plasma (Gooneratne et al., 1995).

In our experimental work, the use of the above-mentioned SPME method in combination with GC-MS produced the following results (Fig. 15.1): maximal concentrations were found in rats 1 h after the poisoning, 2.2 μg/mL in blood plasma and 1.89 μg/g in brain; there was 3−4-fold less FA in rat kidneys, liver, and heart (from 0.64 to 0.50 μg/g). After a further 2 h the distribution between the tissues was more equal, resulting from a prominent decrease of FA in plasma and brain and a small decrease or even elevation of FA in other organs. A further decrease of FA was found in all tissues, except for heart, 24 h after the poisoning. After 72 h, no FA was detected in plasma; we did not measure FA in rat organs at this time point. The half-life was calculated to be 3.6 h.

15.4 Mechanism of action

15.4.1 Molecular mechanism of aconitase inhibition

The mechanism of the inhibitory effect of FA on aconitase [citrate (isocitrate) hydrolyase, EC 4.2.1.3] is one of the most interesting in biochemistry. Upon entering an organism, nontoxic FA undergoes a series of metabolic conversions the result of which is synthesis of highly toxic fluorocitrate (FC); this process is termed

"lethal synthesis" (Peters, 1952). FC is formed by the enzymatic condensation of fluoroacetyl-CoA with oxaloacetate, catalyzed by citrate (*si*)-synthase (EC 4.1.3.7) (Eanes and Kun, 1974). FC was initially considered to be a competitive aconitase inhibitor, but in the early 1990s it was suggested that FC acts as a "suicide substrate," because it has a high affinity for aconitase at any concentration of the competitive citrate (Clarke, 1991). Aconitase effects conversion of citrate to isocitrate through an intermediate, *cis*-aconitate, which binds with aconitase in two different ways, swung 180 degrees to the $C^{\alpha}-C^{\beta}$ bond (Gawron and Mahajan, 1966). Aconitase includes a [4Fe-4S] cluster and the catalytic conversion involves substrate coordination to a specific iron atom in this cluster, Fe_a (Lauble et al., 1992). The single inhibitory isomer was shown to be (−)-*erythro*-2-flurocitrate (2*R*, 3*R*) (Carrell et al., 1970), from which aconitase removes fluoride ion with a stoichiometry of $1F^-$ per enzyme molecule (Tecle and Casida, 1989). The defluorination results in the generation of an actual aconitase inhibitor, 4-hydroxy-*trans*-aconitate (HTA), which binds very tightly—though not covalently—with aconitase (Lauble et al., 1996). The natural aconitase substrate isocitrate should be at a 10^6-fold excess in order to slowly displace HTA from its complex with aconitase. The HTA−aconitase complex involves four hydrogen bonds, which hold together HTA, a water molecule, Asp165, and His167 (Lauble et al., 1994, 1996). In contrast, isocitrate has only one such bond.

15.4.2 Physiological and biochemical effects of fluoroacetate

15.4.2.1 Effects of fluoroacetate and fluorocitrate on mitochondria and other intracellular organelles

Functional disturbances of mitochondria (MCh) precede the appearance of structural anomalies (Buffa and Pasquali-Ronchetti, 1977) and consist of their decreased capacity to oxidize the substrates introduced. Within the mitochondrial matrix, FA induces changes which develop in several minutes, resulting in its swelling and loss of electronic density. These changes are explained by the accumulation of citrate, rise of osmotic pressure, and decrease of energy production; change in the level of ATP is not caused by uncoupling of respiration and phosphorylation (Corsi and Granata, 1967). Mitochondrial volume changes are accompanied with their conformational reorganizations: these are displacement of granules and disintegration of cristae, and extension and rupture of their membranes. Axonal cylinders stretch in 3−4 h after small doses of the poison and in 1−2 h after lethal doses. The cylinders are filled with MCh (most of which is swollen and degenerated), multilamellar lysosome-like bodies,

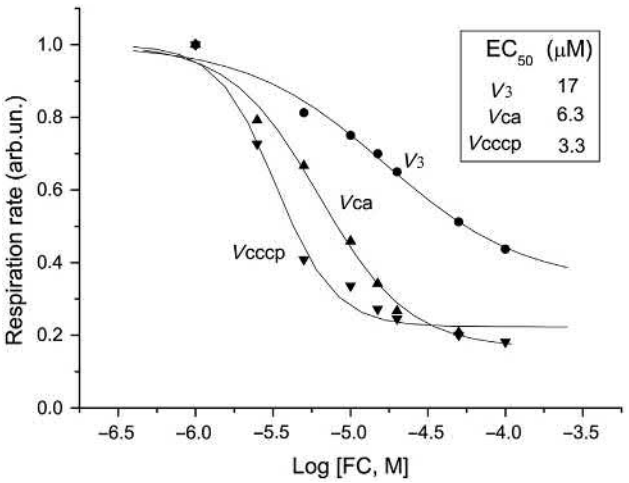

FIGURE 15.2 Effects of FC on respiration of rat liver MCh. Dependence of respiration rate activated by ADP (V_3), calcium transport (V_{Ca}), and protonophore CCCP (*V*cccp) upon concentration of FC. Substrates: pyruvate *plus* malate.

vesicles, and neurofibrils. In the Golgi complex, a condensation of cisternae takes place (McDowell, 1972). Concurrently a disruption of endoplasmic reticulum, swelling of nucleus, and reduction of aggregated chromatin can be seen.

Having studied the in vitro effects of FC on rat liver MCh, we revealed that maximal inhibition of respiration was registered when MCh were uncoupled (Fig. 15.2). The level of alkalinization of the medium at addition of ADP was much lower in the presence of FC, thus evidencing an inhibition of ATP synthesis. The amplitude of alkalinization was also decreased, which could be caused by incomplete ATP synthesis, an additional transmembrane redistribution of protons, and/or change to the binding constant of ADP. FC induced a leak of Ca^{2+} from MCh, which was consistent with the observed inhibition of oxygen consumption in respiratory state 1. Addition of the substrates caused re-entry of Ca^{2+} into MCh. In the presence of FC, the MCh only partially took up the Ca^{2+} ions added to the medium, followed by their spontaneous efflux through an electroneutral $2H^+/Ca^{2+}$ exchanger with $K_{1/2} = 10$ μmol/L (Teplova et al., 1992).

The effects observed under exposure of MCh to SFA developed at much higher concentrations (from 4 mmol/L), as compared to FC, and greatly depended on respiratory substrates. With pyruvate as substrate, the time period of oxidative phosphorylation (OP) and the level of NADH oxidation increased linearly at increasing SFA concentration in the medium (Zinchenko et al., 2007). However, with utilization of succinate and especially glutamate, SFA had no effect on OP in concentrations as high as 8 mmol/L (Fig. 15.3A) and even 16 mmol/L (not shown here). Moreover, the effect of SFA with pyruvate

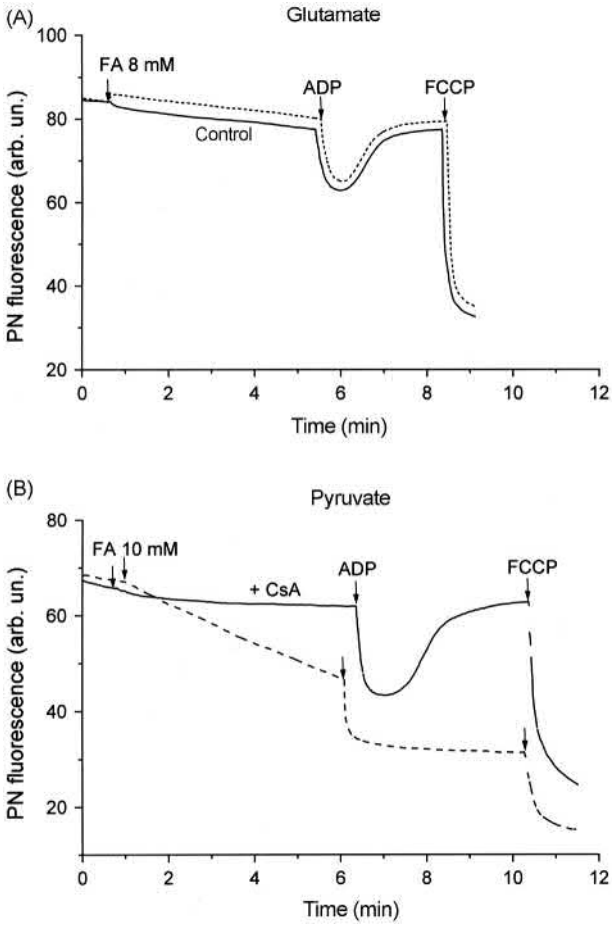

FIGURE 15.3 Effects of FA on redox state of pyridine nucleotides (PN) of rat liver MCh. (A) Glutamate as respiratory substrate. (B) Prevention of PN oxidation and/or leakage by cyclosporin A (CsA) when pyruvate was used as respiratory substrate. Additions: (A) SFA 8 mmol/L (*dots*) or sodium acetate 8 mmol/L (control line), ADP 120 μmol/L, FCCP 1 μmol/L; (B) SFA 10 mmol/L (*dots*) or SFA 10 mmol/L *plus* CsA 1 μmol/L (*line*).

as respiratory substrate can be prevented by incubation of MCh with cyclosporine A, a known inhibitor of the mitochondrial transition pore (Fig. 15.3B). This means that under exposure to FA, development of mitoptosis and apoptosis is possible, but opening of the pore is reversible in nature and preventing oxidation or leak of NADH from MCh can return them back to their normal functional state.

15.4.2.2 Effects of fluoroacetate on isolated cells

The effects of FA on the physiological and biochemical status of cells and tissues are highly dependent upon their level of oxidative metabolism. Thus, FA does not inhibit phagocytosis because of the low level of TCA cycle activity within macrophages (Cifarelli et al., 1979). We investigated a series of cell types—transformed lines and those obtained from animals—under exposure to FA or FC. The level of NAD(P)H in Ehrlich ascite tumor (EAT) cells slowly decreased and the level of Ca^{2+} increased when the cells were incubated with SFA (Zinchenko et al., 2007). SFA obviously induced depletion of intracellular calcium stores and/or activation of influx of extracellular Ca^{2+} ions through the store-operated calcium (SOC) channels. Discovery of other calcium channels, such as TRPV5 and TRPV6 (Hoenderop et al., 2003; van de Graaf et al., 2006), which remain inactivated when cytosolic $[Ca^{2+}]_i$ is increased and become activated when $[Ca^{2+}]_i$ is decreased, stimulated an investigation of the level of calcium ions in endoplasmic reticulum (ER) with chlortetracycline (CTC). FC does not affect the velocity of calcium efflux from ER, so the signal transmission from P2Y receptor via G-protein is not inhibited in EAT cells exposed to FC (Zinchenko et al., 2007). However, FC induced growth in both amplitude of Ca^{2+} leakage and velocity of its influx into ER. A rather long period (8−10 min) of Ca^{2+} influx into ER was observed, which indicated efflux of intracellular Ca^{2+} from cells by plasma membrane Ca-ATPase immediately after mobilization and leaving ER. This greatly reduces $[Ca^{2+}]_i$ for transport back to ER. It was demonstrated earlier (Zinchenko et al., 2001) that the velocity of return transport of Ca^{2+} into ER depends upon the activity of plasma membrane SOC channels. Therefore, we suggest that FA (or FC) somehow impede the entry of calcium ions into cells through SOC channels. This may happen due to chelating of calcium ions by increased extracellular citrate anions leaving the cells through anion channels.

The toxic effects of FC on endothelial cells have been shown to be similar to its effects on other energy-dependent tissues: a reduction of ATP level and oxygen consumption, but an accumulation of lactate and a considerable decrease in protein synthesis (Rist et al., 1996). We have demonstrated with rat aorta endothelial cells in culture a gradual decrease in the mitochondrial membrane potential and elevation of $[Ca^{2+}]_i$ under exposure to SFA, similar to that observed with EAT cells (not shown here). Conversely, in cardiomyocytes SFA induced a slow enhancement of the mitochondrial membrane potential together with a rise of basal $[Ca^{2+}]_i$; propagation of calcium waves along the surface of sarcoplasmic reticulum, or visible elevation and velocity of spreading of the preexisting waves, was also observed (Zinchenko et al., 2007). Probably the increased level of $[Ca^{2+}]_i$ is the reason for its transport into MCh with a subsequent inhibition of the proton ATPase and rise of membrane potential. Mechanistically this phenomenon could be explained by the existence of a Ca^{2+}-dependent protein inhibiting H^+-ATPase (Hubbard and McHugh, 1996).

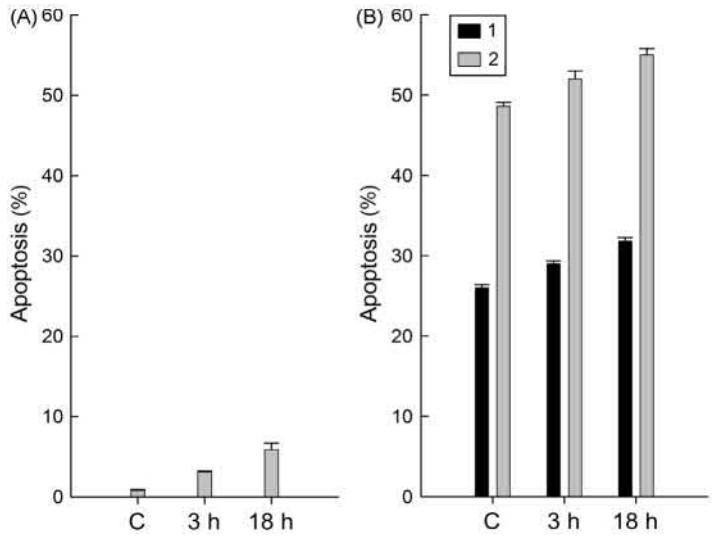

FIGURE 15.4 Effect of SFA on development of apoptosis of rat thymocytes 3 and 18 h after administration of SFA at $1/2LD_{50}$. Registration of apoptosis with Hoechst-33258. (A) Apoptosis in freshly isolated thymocytes; (B) apoptosis in thymocytes cultivating for 20 h after isolation in the absence (1, *black*) and presence (2, *gray*) of dexamethasone. C, control.

We have also studied kinetic parameters of platelet aggregation in experiments with rats in vitro and ex vivo (Mindukshev et al., 2006). Aggregation of platelets was induced with ADP over the concentration range 10−100 nmol/L. The median effective concentrations (EC_{50}) of ADP for the cells exposed to SFA at 10 and 5 mmol/L were calculated to be 25 and 35 nmol/L, correspondingly, and these platelets can be characterized as hypersensitive to ADP. Studying the kinetic parameters of platelet aggregation under intoxication of rats with SFA, we encountered a problem of spontaneous aggregation of the platelet-rich plasma, which is in agreement with the data on primary transition of the platelets to the hypersensitive state. However, the cells that avoided aggregation demonstrated an extremely high level of desensitization. In some experiments, ADP could not induce platelet aggregation at very high (nonphysiological) concentrations near 10 μmol/L. Thrombocytopenia found in cats after the poisoning is in agreement with our observations (Collicchio-Zuanaze et al., 2010).

Under intoxication with SFA a significantly reduced thymus, as well as a prominent quantity reduction of freshly obtained thymocytes and elevation of apoptosis were revealed (Fig. 15.4A). SFA also caused an acceleration of apoptosis of intact and dexamethazone-treated human lymphocytes in vitro (Fig. 15.4B), although spontaneous apoptosis of human neutrophils was inhibited (not shown here). Moreover, SFA either inhibited (high doses) or had little effect on reactive oxygen species (ROS) production by peritoneal macrophages of mice in our experiments in vitro (not shown here). In this regard it should be noted that dexamethasone, in contrast to its effect on lymphocytes, also inhibits apoptosis of neutrophils induced by ROS (Ruiz et al., 2002). One can suggest that the inhibitory effect of FA on neutrophil apoptosis is realized through ROS, whereas the enhanced apoptosis and depression of the cells responsible for adaptive immunity is a nonspecific reaction under SFA intoxication, reflecting a general decline and redistribution of energy resources of the organism. To this end, leucopenia was found in cats after the poisoning (Collicchio-Zuanaze et al., 2010).

15.4.2.3 Biochemical parameters under intoxication with fluoroacetate

Among the biochemical effects caused by FA are: accumulation of citrate and disturbance of its transport from MCh; elevation of lactate and disturbances in carbohydrate metabolism; decrease or increase in free fatty acids (FFA) concentration; elevation of adenosine and ammonia; imbalance of bivalent cations and acid−base equilibrium; hypokalemia; changes in GABA balance in brain; hypo- or hyperphosphatemia; and rise of various enzymes in blood plasma, including creatine kinase enzyme and creatine kinase cardiac isoenzyme (Buffa and Peters, 1950; Engel et al., 1954; Maytnert and Kaji, 1962; Stewart et al., 1970; Buffa et al., 1973; Liang, 1977; Taitelman et al., 1983a; Bosakowski and Levin, 1986; Szerb and Redondo, 1993; Tsuji et al., 2009; Collicchio-Zuanaze et al., 2010). We have specified changes in triglycerides and FFA after acute intoxication with SFA (Table 15.1). As expected, the total level of triglycerides and FFA changed in opposite directions within 3 h after poisoning, with the former reduced and the latter elevated up to 75%. Reduced level of triglycerides persisted over 24 h, whereas the total concentration of FFA dropped below the normal level, though not significantly. GC-MS analysis of 36 fatty acids after extractive alkylation revealed reliable changes in concentration for seven of them, in agreement with total FFA changes.

TABLE 15.1 Triglycerides and FFA concentrations in rat blood plasma in normal state and after introduction of SFA, $1/2LD_{50}$.

	Triglycerides	Free fatty acids							
		Total	Dodecanoate, C12:0	Palmitoleate, C16:1n7	Palmitate, C16:0	Linoleate, C18:2n6c	Oleate, C18:1n9c	Elaidate, C18:1n9t	*cis*-4,7,10,13,16,19-Docosahexaenoic acid methyl ester, C22:6n3
	mM	μM	μg/mL						
Control	1.62 ± 0.24	100.6 ± 13.6	0.49 ± 0.18	5.3 ± 1.1	33.3 ± 5.8	50.2 ± 14.0	13.5 ± 6.1	2.1 ± 0.6	2.0 ± 0.9
3 h	0.96 ± 0.11*	174.6 ± 9.46*	0.88 ± 0.24*	7.7 ± 2.4	49.3 ± 11.2*	76.4 ± 16.0*	20.9 ± 4.2	3.6 ± 1.1**	3.6 ± 1.0*
24 h	0.98 ± 0.13*	82.4 ± 16.8	0.66 ± 0.29	4.5 ± 1.8	28.0 ± 11.2	47.6 ± 23.9	8.6 ± 6.9	1.4 ± 0.6	1.4 ± 0.7

*$P < .05$, **$P < .01$.

Among such a variety of biochemical changes, citrate seems to be the only parameter whose qualitative (but not quantitative) trends are not a matter of controversy. In rat hearts under acute intoxication with FA, the concentration of citrate can exceed control values by 8−15-fold (Bosakowski and Levin, 1986). Elevation of citrate concentration is in direct proportion to the respiratory activity of a tissue: metabolically active tissues—such as heart, kidneys and spleen—maximally accumulate citrate. However, in liver, which is also characterized by high respiratory level and metabolic activity, a small accumulation of citrate has been observed (Cole et al., 1955; Twigg et al., 1986). In our experiments with rats poisoned with SFA at a dose $1/2LD_{50}$, the concentrations of citrate in blood plasma and organs increased within an hour (Fig. 15.5). The most prominent elevation of citrate was revealed 6 h after the poisoning in the heart (fivefold), kidneys (threefold), and brain (2.5-fold). There was a doubling of the level of citrate in blood plasma after an hour, though it was the only biochemical parameter of plasma that remained elevated for 3 days.

Transfer of citrate through the inner membrane of MCh is provided by a tricarboxylate transporter (molecular weight 32.5 kDa), which also catalyzes transport of *treo*-D_s-isocitrate, *cis*-aconitate, and other tricarboxylates (Kaplan et al., 1990). This is electroneutral exchange for either another tricarboxylate or dicarboxylate (e.g., malate or succinate), or for phosphoenolpyruvate. Formation of glutathione-citryl thioester is irreversibly inhibited by (−) erythrofluorocitrate ($IC_{50} = 25$ pmol FC/mg protein), which makes a stable adduct with the synthase (Kun et al., 1977). However, the block of citrate transport is not absolute and universal for all organs and tissues. There are data on citrate transfer from MCh to cytosol with its subsequent utilization by cytoplasmic aconitase (c-aconitase), which is virtually unaffected under FA intoxication, and then by cytoplasmic NADP-dependent isocitrate dehydrogenase (cICDH) (Max and Purvis, 1965). Around 32% of citrate produced in MCh can be transported to cytosol (Buffa et al., 1972). These processes should be regarded as adaptive and positive, they lead to reduced oxygen consumption because the NADPH generated does not require further oxidation in the respiratory chain and can be utilized in other metabolic pathways.

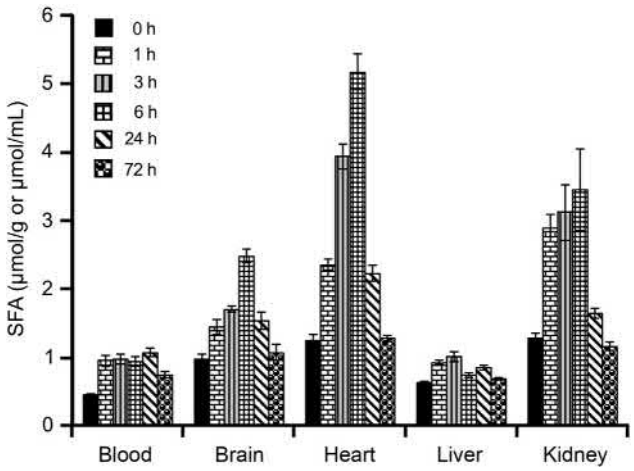

FIGURE 15.5 Concentration of citrate in blood and organs of rats under intoxication with SFA at $1/2LD_{50}$.

Among the negative consequences of citrate accumulation is a change of electrolyte composition and acid−base imbalance in the organism. Moreover, elevation of the citrate level in cells leads to a disturbance of glucose metabolism due to inhibition of the key glycolytic enzyme phosphofructokinase (Bowman, 1964; Peters, 1972). Hyperglycemia during intoxication with FA can be very prominent, in spite of inactivation of gluconeogenesis (Godoy et al., 1968; Bobyleva-Guarriero et al., 1983). Nevertheless, we could not find significant changes in rat blood glucose throughout the periods of intoxication with FAA or SFA at a dose $1/2LD_{50}$; at the same time,

there was a significant increase of glucose level in the liver, heart, and brain (not shown here). This may signify a utilization of glucose by other tissues, and first of all by skeletal muscles, as a result of which the local increase of glucose in organs is not reflected by the level of glucose in blood. Thus, glucose cannot serve as a reliable criterion of intoxication.

Some researchers considered the elevated glucose level to be a result of decreased insulin secretion by pancreatic β-cells due to their damage by FA (Cole et al., 1955; Karam and Grodsky, 1962). Along with hyperglycemia, there was hyperketonemia, observed characteristically for the diabetic state, caused by inhibition of TCA cycle and depletion of oxaloacetate (Buffa et al., 1973; Taitelman et al., 1983a). Also consistent with diabetes is inhibition of hormone-induced lipolysis in adipose tissue (Taylor et al., 1977). Moreover, FA increased glucose conversion to fatty acids, and such a coincidence of antilipolytic and lipogenetic effects of FA provides a basis for suggesting a relation in effects of FA and insulin. However, injection of insulin does not alleviate FA intoxication in general and "FA diabetes" in particular (Reichelt, 1979). During FA intoxication, the initial hyperglycemia can even be reversed into hypoglycemia (Boquist et al., 1988), therefore this effect of FA was considered to be an insulin-like phenomenon (Zieve et al., 1983). The principal distinction, however, should be depletion of glycogen stores in different tissues under intoxication with FA (Godoy et al., 1968; Boquist et al., 1988). After poisoning with FA, glycogen levels in animal tissues may decrease by 75% in 1 h and by 90% in 2 h (Buffa et al., 1973; Zhou et al., 1984). According to our data, during SFA intoxication ($1/2LD_{50}$) glycogen levels are maximally decreased after 6 h in both liver (by 55%) and brain (by 40%), and the dynamics of the glycogen levels were similar in these organs. Such a decrease could result from the indirect action of adrenaline or sympathetic regulation (Buffa et al., 1973). In addition, inhibition of de novo glycogen synthesis has been reported (Zhou et al., 1984).

Nevertheless, disturbances in hormonal regulation during FA intoxication can also take place: reduction of calcium concentration in blood plasma could be caused by a poor reabsorption of calcium ions in kidneys due to a decrease in the parathormone level; an excess of Ca^{2+} excretion up to 0.173 mg/min (the control rate being 0.06 mg/mL) has been registered (Perez and Frindt, 1977). A decrease in the calcium level could be the cause of so-called "hypocalcemic tetanus" (Roy et al., 1980), manifesting itself as typical convulsions, disturbances of blood clotting, and hypotension leading to vascular attacks. The level of decrease of calcium correlates with extension of the $Q-T$ interval on ECG, which is a consequence of the broad spectrum of cardiac arrhythmia (Buffa and Peters, 1950; Arena, 1970).

The ATP level is usually reduced, and ADP and AMP levels can be elevated in the first hours of the FA intoxication, with a subsequent decrease (Bowman, 1964; Stewart et al., 1970). There are other reports of a constant level of ATP in some organs and tissues. For example, FA did not affect ATP and GTP, as well as cyclic nucleotides levels in hepatocytes in vitro (Dohi and Murad, 1981). When dogs were intoxicated with sublethal doses of FA, there were no decreases in oxygen consumption and ATP level observed, which was explained by utilization of glutamate and aspartate which can enter the TCA cycle distally of aconitase (Liang, 1977). The inversion of reactions at glutamate dehydrogenase (GDH) is a simple and effective compensative mechanism during blockade of the TCA cycle in kidney cells: instead of glutamine synthesis, glutamate is deaminated to form 2-oxoglutarate to support the flow of reducing equivalents in the TCA cycle and ATP synthesis, while the ammonia produced neutralizes local tissue acidosis (Yu et al., 1976). Such utilization of glutamate may account for the significant reduction of glutamate level in rat organs, beginning from the first hour after intoxication with FA. The data obtained according to the GC-method of Matsumura et al. (1996) have also shown decreases in glutamate, aspartate, and some other amino acids in rat brain (Fig. 15.6), as well as a decrease in glutamate and nearly complete absence of glutamine in the blood plasma of rats and rabbits (not shown here), 3 h after poisoning with SFA. The levels of amino acids in blood plasma of animals indicate the extent of protein breakdown in muscles, on one hand, and the level of their utilization by other organs and tissues, on the other hand. Under intoxication with FA, glutamate and its precursor glutamine are probably the main

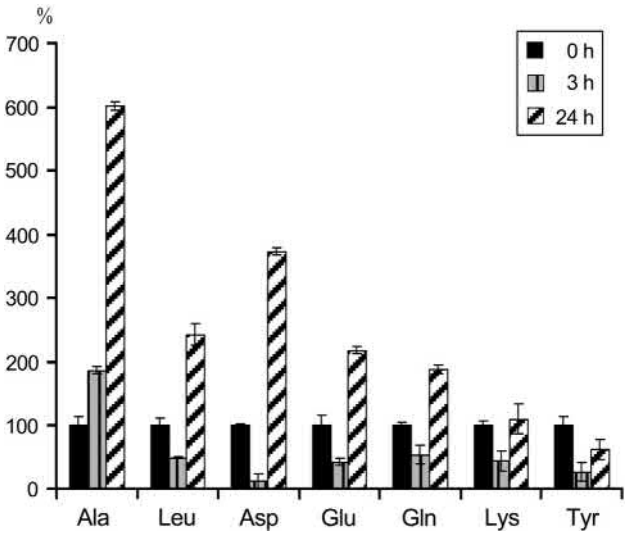

FIGURE 15.6 Changes to some amino acids in rat brain 3 and 24 h after administration of SFA at $1/2LD_{50}$.

nutrients. Elevation of amino acid levels in blood plasma of rats within 3 h after poisoning signifies an elevation of protein breakdown. Furthermore, this indicates that other amino acids—because of their transport, catabolism, etc.—are not nutrients of primary importance under energetic deficit conditions.

Elevation of the lactate level in the blood of animals poisoned with FA has been reported (Engel et al., 1954; Taitelman et al., 1983a). In agreement with these data, we observed a prominent rise in lactate levels in blood just after convulsions (not shown here). In rat heart and brain, lactate levels decreased under intoxication with SFA or FAA, irrespective of convulsions. During SFA intoxication the decrease in lactate level (and increase in glucose level) in rat heart takes place earlier and to a greater extent than for FAA intoxication: 38% decrease in 3 h for lactate, as compared with 25% in 6 h in the case of FAA; 100% increase in 3 h for glucose, as compared with 67% in 6 h in the case of FAA. Also, the maximal increase in citrate was registered at 24 h after poisoning with FAA, but at 6 h after poisoning with SFA. These and other biochemical data are consistent with clinical pictures of intoxication with equipotential doses of SFA and FAA: intoxication with SFA is generally more violent and takes a shorter period of time.

15.4.2.4 Effects of fluoroacetate on the cells of the nervous system: interaction of glia and neurons

Acetate is metabolized in astrocytes near 18-fold faster than in cortical synaptosomes, though activity of acetyl-CoA synthase in synaptosomes is almost double that in astrocytes (5.0 and 2.9 nmol/min mg/protein, respectively). The principal difference in the acetate metabolism rates is explained by differences in the kinetics of its transport, which is mediated by a monocarboxylate carrier (Hosoi et al., 2004); acetate uptake by astrocytes, unlike synaptosomes, rapidly increases and follows saturation kinetics ($V_{max} = 498$ nmol/min/mg protein, $K_M =$ 9.3 mmol/L) (Waniewski and Martin, 1998). Having penetrated into astrocytes at one site, FA can diffuse into other cells through gap junctions (Ransom, 1995). Citrate accumulating in astrocytes is readily released from cells and effectively penetrates other astrocytes (Westergaard et al., 1994). The TCA cycle in nerve tissues is blocked by FA but not completely, only by 35%−55% (Patel and Koenig, 1968). This leads to decreased consumption of glucose and increased consumption of glutamine (if the latter is available); no reduction of ATP was observed (Hassel et al., 1994). The natural metabolic pathway is switched over to utilization of glutamine, glutamate, and 2-oxoglutarate in the TCA cycle. GDH of astroglia plays a large role in this switching over, promoting the ATP-independent utilization of glutamate (Plaitakis and Zaganas, 2001). The absence of an aspartate/glutamate mitochondrial exchanger (the key component of the malate/aspartate cycle) in astrocytes also plays a part in support of this (Xu et al., 2007). There is little GDH in neurons as compared to astrocytes, with activity of GDH depending not only upon proximity to glutamatergic fibers and terminals, but also upon the activity of neighboring neurons regardless of their functional specialization; a deficiency in GDH activity in astroglia may be a cause of cytotoxic effects of glutamate and aspartate (Aoki et al., 1987).

The uptake of glutamate by astrocytes is an electrogenic process in which one molecule of glutamate is cotransported with three sodium ions (or $2Na^+$ and $1H^+$), being exchanged for $1K^+$ and $1OH^-$ or $1HCO_3^-$ (Bouvier et al., 1992). To re-establish the ionic balance, Na^+/K^+-ATPase would work with ATP provided by phosphoglycerate kinase bound to plasma membrane. This stimulates glycolysis and lactate production in astrocytes. Lactate is released from astrocytes and then is taken by neurons to be further oxidized. Pyruvate, which is also produced in astrocytes can be utilized in the TCA cycle to form 2-oxoglutarate or transaminated to form alanine; the latter can also enter neurons (Tsacopoulos and Magistretti, 1996; Tsacopoulos, 2002). However, the rate of alanine metabolism through alanine transaminase (ALT) in synaptosomes is much less than its rate of uptake. Moreover, neuronal ALT and AST work mainly to synthesize alanine and aspartate (Erecinska and Silver, 1990). Hence, we observed a stable elevation of alanine level—in contrast to that of other amino acids—in brains of rats poisoned with SFA (Fig. 15.6). The role of alanine as a source of glutamate is increased during the restoration period after ischemia/hypoxia, when the alanine concentration is elevated and the glutamate concentration is reduced (Erecińska et al., 1994).

Inhibition of glutamate uptake by astroglial cells can be one of the causes of convulsions observed under intoxication with FA (Szerb and Issekutz, 1987). The toxic effect is governed mainly by citrate, which is thought to chelate calcium ions (Fonnum et al., 1997). Intrathecal injection of FC in mice caused convulsions in about 15 s, and about 37 min by intracerebroventricular injection (Hornfeldt and Larson, 1990). Moreover, intrathecal injection of sodium citrate caused the same effect. This means that the main target of FC and citrate, and the area for generation of convulsions, should be the spinal cord. Convulsions could also be generated by other compounds having the common property of chelating calcium ions; these are EDTA, EGTA, glutamate, and lactate (Hornfeldt and Larson, 1990). Thus, along with elevation of citrate level, activation of anaerobic oxidation of glucose in neurons followed by accumulation of

lactate in cerebrospinal fluid, could also lead to coma and convulsions (Stewart et al., 1970). Chelation of zinc and other divalent cations by citrate enhances the signaling activity of NMDA receptors (Westergaard et al., 1995). In addition, disturbances of GABA metabolism were revealed as a result of the TCA cycle blockade: after injection of FA, there was initially an elevated level of GABA registered in different regions of the brain, followed by its reduction concurring with the beginning of clonic-tonic convulsions (Maytnert and Kaji, 1962; Stewart et al., 1970).

The convulsive state is aggravated by an increasing concentration of ammonia ions (Raable, 1981), an excess of which can lead to redistribution of K^+ and Cl^- ions, disturbances of neuronal depolarization and hyperpolarization, and impairment of postsynaptic inhibition. The neuron dysfunctions observed result in encephalopathy, ataxia, convulsions, and coma (Iles and Jack, 1980; Raable and Lin, 1983; Xiong and Stringer, 1999). Normally, ammonia specifically promotes GSH synthesis and export from astrocytes and increases its extracellular degradation, which may improve the availability of precursors for GSH synthesis in neurons and their resistance to ammonia toxicity; FA abrogates this defense mechanism (Hilgier et al., 2010). Lymphocyte-mediated neuroprotection is also mediated by astrocytes and there is no wonder it is inhibited by FA (Shrestha et al., 2014). Moreover, FA impairs enhancement of synaptic transmission and facilitation of long-term memory caused by nicotine, the effect being mediated by the gliotransmitter D-serine, an endogenous co-agonist of NMDA receptors (López-Hidalgo et al., 2012). On the other hand, FC affecting astroglia cause a drop in membrane potential and depolarization, and decrease of $[K^+]_i$ (Largo et al., 1997); this should lead to compensatory transport of bicarbonate ions into astrocytes and acidification of the extracellular medium. Together with the natural carbonate acidification of the medium close to chemoreceptors of the retrotrapezoid nucleus, this activates the diaphragmal nerve and increases the expired minute ventilation (Erlichman et al., 1998; Holleran et al., 2001): maximum ventilation is attained at 4% CO_2 against 8%−10% in control hypercapnic trials. Control of extracellular pH in nervous tissue is coupled with functioning of the Na^+/HCO_3^- cotransporter, existing in plasma membrane of astrocytes but lacking in that of neurons (Deitmer, 1992; Romero and Boron, 1999). This transport has an electrogenic character, because two or even three bicarbonate ions are transferred per one sodium ion. Again, however, a continuous supply of glutamine to the glutamatergic nerve terminals is the necessary condition of respiratory rhythm generation; blockade of the TCA cycle in astroglial cells with FA can impair the respiratory activity (Hulsmann et al., 2000).

15.4.3 Physiology of blood vessels under intoxication with fluoroacetate

FA does not affect circulation in resting organs, but a significant increase of blood flow can be seen in working respiratory muscles (Johnson and Reid, 1988). Conversely, a reduction of blood was registered in the hepatic artery, and contractive activity of isolated portal veins was suppressed after introduction of FA into the medium (Liang, 1977). These data, along with data on the effects of FA on endothelial cells in vitro, suggested that the endothelium of blood vessels could be one of the primary targets for FA. If so, the endothelium-dependent relaxation of blood vessels would be affected. To test the hypothesis, we administered SFA to rats subcutaneously at a dose of 2−3 mg/kg (LD_{50}−LD_{84}), and investigated endothelium-dependent relaxation of rat aorta 3 and 24 h after the poisoning. Norepinephrine in saturating concentrations induced a rapid constriction of the aorta followed by a smooth transition to plateau; in contrast, vasoconstricting hormones angiotensin II, vasopressin, and 5-hydroxytryptamine induced a bell-shaped vasoconstricting response of aorta. To assess the functional state of endothelium, carbocholine was introduced at 10^{-5} mol/L. Acting on muscarinic receptors of endothelial cells, it induced the generation of nitric oxide and release of endothelium-derived hyperpolarizing factor (McCulloch et al., 1997). All the agonists applied had similar effects on contraction of aortas obtained from control and poisoned animals (not shown here). The experiment clearly demonstrated that FA has no influence upon the contractile properties of isolated rat aorta at 3 and at 24 h after poisoning. This endothelial function is not affected, at least not directly, under intoxication with FA.

15.4.4 Body temperature of rats and rabbits under intoxication with fluoroacetate

One of the main pathophysiological features of intoxication with FA is a decrease in the body core temperature of endotherms, which indicates a disturbance of heat production and/or regulation (Brockmann et al., 1955; Misustova et al., 1980; Taitelman et al., 1983b). It is interesting to note that the effects of FC are comparable with those of selective inhibitors of p38 MAP-kinases (activation of which precedes production of pyrogens) and antagonists of cytokines TNF, IL-1, and IL-6 (Milligan et al., 2001, 2003). In our experiments, following administration of lethal doses of SFA to male rats, a marked decrease in rectal temperature was registered beginning from 1 h after the poisoning and gaining minimal levels in 6 or 24 h depending on the dose. Then a gradual increase in temperature took place in surviving rats, returning to normal in 2−7 days. For example, under

intoxication with SFA at a dose LD_{50}, a minimal rectal temperature of the surviving male rats (31.5°C as compared to 38.5°C in control animals) was registered in 6 h, and 7 days after the poisoning the temperature was 1°C below the control level. Under intoxication with FAA at equipotential doses, a minimal rectal temperature of the surviving male rats (32.6°C as compared to 38.9°C in control animals) was registered in 2 days, and even 7 days after the poisoning the temperature was 3°C below the control level. However, we observed much less change in rectal temperature in rabbits after s.c. administration of SFA at a dose LD_{50}: maximal decrease was only 1°C (38°C as compared to 39.1°C) in 6 h. According to our observations, a decrease in the temperature below 38°C in 3 h serves as a reliable sign forecasting the lethal outcome of the poisoned rabbits.

15.4.5 Electrophysiological studies of fluoroacetate intoxication

Clinical analysis of ECG of rats poisoned with SFA or FAA (Kuznetsov et al., 2007) revealed a similar dynamic of the temporal parameters of ECG, with slowing down and delay of the repolarization processes being the most important (Table 15.2). A drop in amplitudes of the atrial and ventricular ECG complexes can be observed within an hour after poisoning, followed by a decrease in the systolic index in 24 h, thus indicating an impairment of the contractile capacity of myocardium. Registration of ECGs of rats which died within 2 days of intoxication with SFA revealed a sharp drop in the heart rate (down to 120−180 per min) 24 h after the poisoning, together with complete absence of the P wave, which reflects atrial depolarization (Fig. 15.7A). Simultaneous reduction of both the amplitude and duration of the T wave can be seen. An upward shift of the ST segment, though not accompanied by growth of the T wave amplitude, was registered in 70% of rats (Fig. 15.7B). The cumulative evidence of the shape and amplitude changes of the ECG waves indicates the development of acute myocardial ischemia, though this was transient and maximally expressed 24 h after the poisoning. Reduction of the S wave amplitude could be caused by disturbances of excitation processes in basal ventricular regions and in some areas of the right ventricle. Taking into account an increase in the duration and shape distortions of the ventricular complex, one cannot exclude an incomplete right bundle-branch block. A significant extension of the T wave during the course of examination is indicative of deceleration of the fast repolarization of myocardium, though the process of slow repolarization (the QT interval in ECG, corresponding to the systole of ventricles) is accelerated within 3−24 h after the poisoning.

Respiratory rhythm was gradually increased in rats under intoxication with SFA, and there were additional respiratory components in 50% of animals 24 h after administration of the poison (Fig. 15.8) which may indicate disturbances of innervation of respiratory muscles. Spectral analysis of the respiratory curve demonstrated that there was an enhanced synchronization of the respiratory rhythm observed within 3 h after the poisoning. Simultaneously, the amplitude of respiration increased followed by a gradual decrease to the third day. Over the same period, a certain reduction in the lability of respiratory rhythm was noted, accompanied by the appearance of two distinct peaks corresponding with frequencies of 90 and 120 cycles of respiration per minute. By the seventh day, the respiratory spectrogram was similar to the initial one, though the frequency of respiration was not completely restored. Comparison of spectrograms of respiration and ECG demonstrates disturbances of control mechanisms underlying generation of the second-order waves (respiratory arrhythmia visible at the spectrogram as a peak in the high-frequency region 0.8−2.5 Hz). One day after administration of SFA, there was a marked frequency maximum at the respiratory spectrum, in contrast to that of the ECG spectrogram.

Analysis of the heart rate variability (HRV) demonstrates that 1 h after poisoning an enhancement of parasympathetic influence took place, and this was accompanied by insignificant and paradoxical enhancement of heart and respiratory rates (Table 15.3). Then, against a background of enhancement of humoral (metabolic) and sympathetic influences and simultaneous decline of parasympathetic influence, a stable decrease in heart and respiratory rates took place, indicating a prominent divergence between vagosympathetic balance and resulting physiological parameters. Previously it was shown in experiments with dogs that systemic, pulmonary, and coronary hemodynamic parameters during the first hours after introduction of FA were not mediated by the autonomic nervous system and adrenergic neuromediators (Liang, 1977). This is in partial agreement with our results obtained with rats, though this cannot be extrapolated to all the periods of intoxication and all animal species.

15.5 Toxicity and risk assessment

A characteristic feature of the clinical picture of intoxication with FA is a latent period of 0.5−6 h (Egekeze and Oehme, 1979). The duration of the latent period depends on animal species' metabolism and dose administered (Chenoweth, 1949; Goncharov et al., 2006). A broad variability of clinical manifestations of FA effects in different animal species is one of its characteristic features. There is a correlation between food specificity and the toxic

TABLE 15.2 Parameters of ECG (averaged cardiocycle) of adult rats in normal state and different terms after introduction of SFA, 1/2LD_{50}.

Terms	Parameters										
	Amplitude (mV)				Duration (s)						SI
	P	R	S	T	P	T	PQ	QRS	QT	RR	
Background	0.297 ± 0.019	0.973 ± 0.131	0.723 ± 0.137	0.747 ± 0.071	0.018 ± 0.001	0.036 ± 0.001	0.050 ± 0.001	0.019 ± 0.001	0.057 ± 0.001	0.140 ± 0.003	40.7
1 h	0.144 ± 0.016***	0.669 ± 0.083	0.235 ± 0.088*	0.469 ± 0.089*	0.018 ± 0.001	0.049 ± 0.002***	0.050 ± 0.001	0.022 ± 0.002	0.070 ± 0.002***	0.139 ± 0,005	50.4
3 h	0.170 ± 0.017***	0.826 ± 0.066	0.161 ± 0.059**	0.518 ± 0.083	0.021 ± 0.001*	0.044 ± 0.002**	0.057 ± 0.004	0.022 ± 0.001*	0.067 ± 0.002***	0.182 ± 0.011**	36.8
1 day	0.124 ± 0.030***	1.153 ± 0.171	0.089 ± 0.050**	0.538 ± 0.143	0.019 ± 0.001	0.051 ± 0.002***	0.048 ± 0.002	0.024 ± 0.002*	0.075 ± 0.004***	0.237 ± 0.036*	31.7
3 days	0.195 ± 0.023**	1.309 ± 0.122	0.102 ± 0.052**	0.403 ± 0.102**	0.019 ± 0.004	0.046 ± 0.003**	0.052 ± 0.004	0.025 ± 0.001**	0.071 ± 0.003***	0.158 ± 0.005**	44.9
7 days	0.167 ± 0.012***	1.106 ± 0.113	0.450 ± 0.088	0.458 ± 0.041**	0.018 ± 0.001	0.062 ± 0.003***	0.052 ± 0.002	0.025 ± 0.001**	0.088 ± 0.003***	0.171 ± 0.007***	51.5

*$P < .05$, **$P < .01$, ***$P < .001$.*SI*, Systolic index, calculated after formula SI = (QRST*100)/RR.

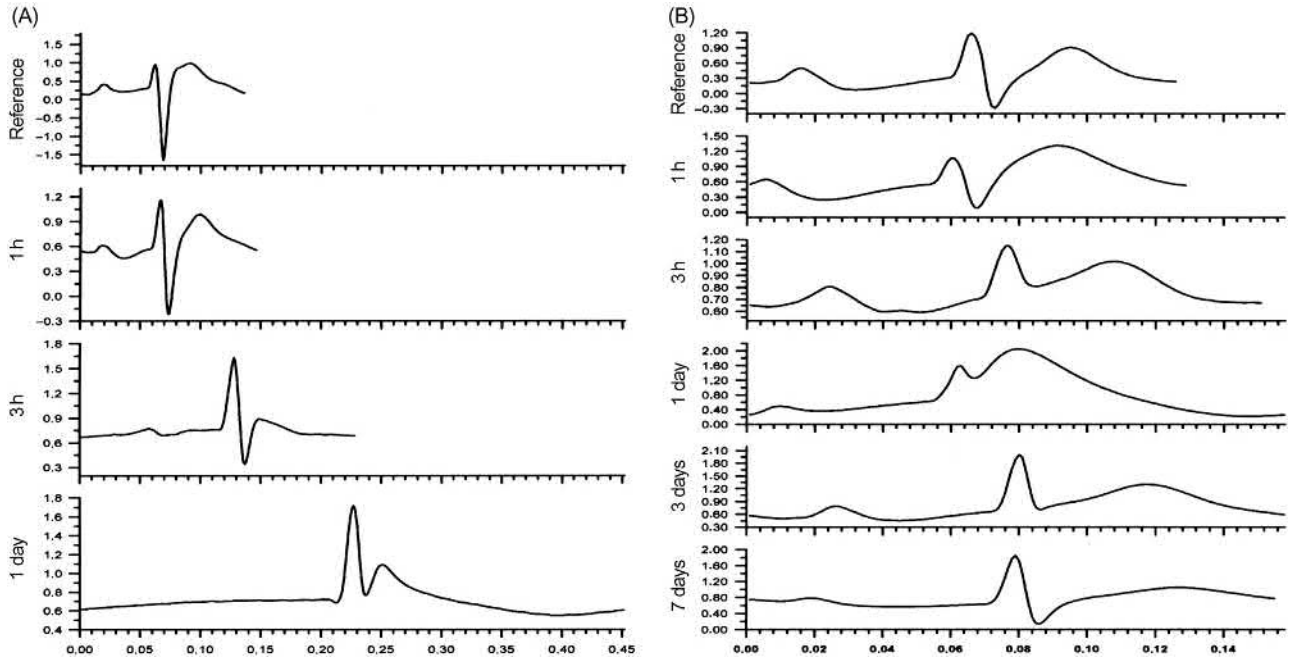

FIGURE 15.7 (A) ECG (averaged cardiocycle) of a rat that died almost 2 days after introduction of SFA at LD_{50}. (B) ECG (averaged cardiocycle) of a rat that survived after introduction of SFA at LD_{50}. Along the *X*-axis, time (s); along the *Y*-axis, amplitude (mV).

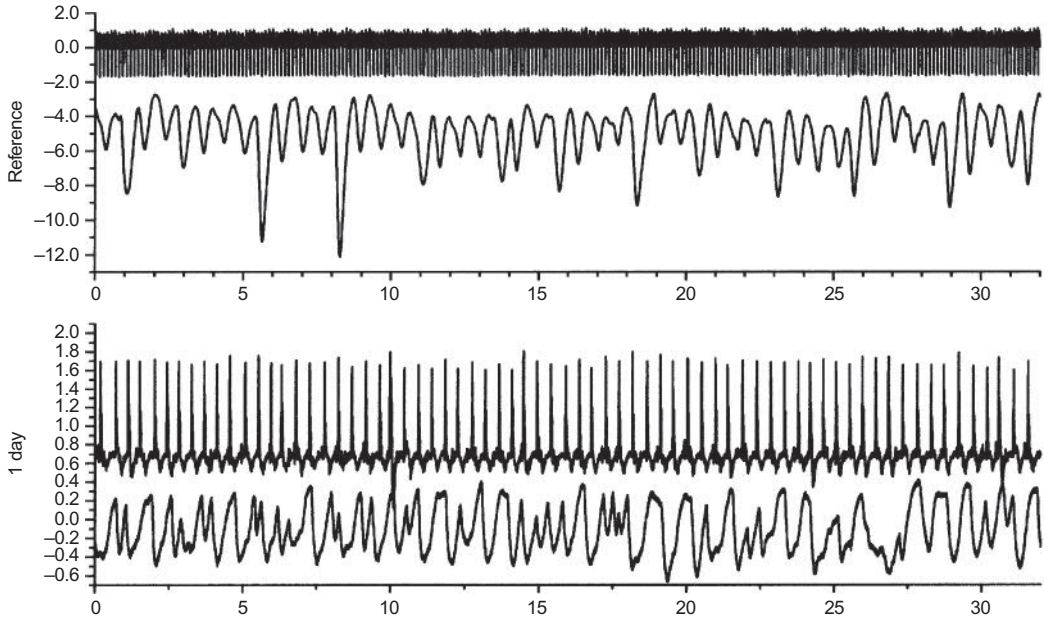

FIGURE 15.8 Records of ECG (upper) and respiratory rhythm (lower) from a narcotized rat before and 24 h after introduction of SFA.

effect of FA; the cardiovascular system is mainly affected in herbivores, while the CNS is mainly affected in carnivores. Accordingly, four groups were recognized in terms of clinical signs of intoxication (Chenoweth and Gilman, 1946). The first comprised herbivores (rabbits, goats, sheep, cattle, and horses), in which FA induced ventricular fibrillation without notable CNS disorders (Marais, 1944; Chenoweth, 1949; Egekeze and Oehme, 1979). The second group comprised dogs and guinea pigs, in which the CNS was primarily affected. In dogs, a species highly sensitive to FA, symptoms of secondary intoxication appear after a latent period of 1–10 h (Chenoweth and Gilman, 1946; Egyed and Shupe, 1971). For animals of the third group the clinical pattern of intoxication is

TABLE 15.3 Analysis of heart rate variability of adult rats in time and frequency domains under intoxication with SFA at $1/2LD_{50}$.

Parameters	Period of examination											
	Control		1 h		3 h		24 h		3 days		7 days	
	Value	Shift (%)	Value	Shift (%)	Value	Shift (%)	Value	Shift (%)	Value	Shift (%)	Value	Shift (%)
Heart rate (contr/min)	424.6 ± 6.1	–	441.6 ± 9.4	+4.0	340.1 ± 11.4***	−20.0	291.8 ± 18.5***	−31.3	370.6 ± 6.0***	−12.7	341.0 ± 6.0***	−19.7
Coefficient of arrhythmia (rel. un.)	0.049 ± 0.003	–	0.058 ± 0.005	+18.4	0.156 ± 0.042*	+218.4	0.245 ± 0.073*	+400	0.053 ± 0.003	+8.2	0.051 ± 0.006	+4.0
Value of VLF (ms^2)	0.041	8.8	0.053	7.6	0.087	11.8	0.060	9.7	0.043	10.1	0.044	10.9
Value of LF (ms^2)	0.075	16.1	0.107	15.2	0.128	17.3	0.114	18.4	0.073	17.1	0.079	19.6
Value of HF (ms^2)	0.350	75.1	0.542	77.2	0.523	70.9	0.445	71.9	0.311	72.8	0.280	69.5
Value of To (ms^2)	0.466	–	0.702	+50.6	0.738	+58.4	0.619	+32.8	0.427	−8.4	0.403	−13.5
LF/HF (rel. un.)	0.214	–	0.197	−7.9	0.245	+14.5	0.256	+19.6	0.235	+9.8	0.282	+31.8
HF/To (rel. un.)	0.751	–	0.772	+3.8	0.709	−5.6	0.719	−4.3	0.728	−3.1	0.695	−7.5
Respiration rate (min)	94.1 ± 3.0	–	101.9 ± 3.1	+8.3	94.4 ± 3.2	+0.3	98.8 ± 3.7	+5.0	111.9 ± 6.1*	+18.9	120.2 ± 8.1**	+27.7

*$P < .05$, **$P < .01$, ***$P < .001$.
Coefficient of arrhythmia = $(RR_{max} - RR_{min})/RR_{mean}$.
Values of shifts (%) for VLF, LF, and HF indices are given against To index of corresponding period of examination. For other parameters the shift was calculated against the initial control value.

similar to that of the second group of animals, but slightly less pronounced. This group comprised rats and hamsters relatively tolerant to FA. After a latent period lasting 1−2 h, tremor and elevated excitability were common symptoms. Death usually occurred within 4−6 h as a result of respiratory depression, after exposure to high FA doses (Chenoweth and Gilman, 1946; Pattison, 1959). The surviving animals demonstrated depression, weakness, ataxia, and strongly pronounced bradycardia down to 30 heartbeats per minute. At sublethal doses of FA, full recovery can occur in 72 h after poisoning (Chenoweth and Gilman, 1946; Pattison, 1959). A mixed response to FA exposure was described in animals of the fourth group—cats, pigs, and rhesus monkeys; it included disturbances of both the CNS and the cardiovascular system. On acute poisoning, adynamia, salivation, vomiting, frequent defecation, pupil dilatation, nystagmus, accelerated respiration, enhanced excitability, tremor, and clonic-tonic convulsions were observed in these animals (Chenoweth, Gilman, 1946; Gammie, 1980). In cats, lesions were observed characteristic of degenerative and ischemic processes in the heart, kidneys, liver, brain, and lungs (Collicchio-Zuanaze et al., 2010).

Nevertheless, the classification outlined above was criticized (Sherley, 2004). The division of animals into cardiac and neurological symptomatic groups is considered to be unnatural as it ignores common neurological signs manifested in all the groups: among these are tremor, ataxia, hypersensitivity, myotonic convulsions, weakness, and partial paralysis. The cardiac response in a pure form was not a common event and was described just for a limited number of animals, though CNS involvement is obviously widespread.

As for humans, exposure to stock solution during formulation and dermal or respiratory exposure during application of baits, as well as accidental or intentional acute intoxications, are the main human health concerns. Formulators and pest control workers are the largest occupational risk group (Norris, 2001; Beasley et al., 2009). Early monitoring indicated exposures were highest in relation to cereal bait manufacturing and aerial carrot baiting procedures (Beasley et al., 2009).

The clinical picture of acute intoxication of humans is similar to that of rhesus monkeys, and among the symptoms are nausea, vomiting, abdominal pains, salivation, irrational fear, weakness, tachypnea, cyanosis, and sometimes sweating and increased temperature (Brockmann et al., 1955; Pattison, 1959; Arena, 1970; Taitelman et al., 1983b). Psychomotor agitation and sometimes a loss of spatiotemporal feeling can occur. In addition, tremor, nystagmus, involuntary defecation and urination, muscle spasms, hypertonus of the extremities, and even alalia, have been reported (Gajdusek and Lutheer, 1950; Robinson et al., 2002). The most characteristic signs of intoxication involve generalized recurrent convulsions alternating with deep depression. Sudden loss of consciousness and coma may occur. These symptoms were associated with metabolic acidosis and hypotension (Pattison, 1959; Chi et al., 1996, 1999), as well as cardiac rhythm disturbances, such as tachycardia, bradycardia, asystolia, and sustained ventricular fibrillations (Gajdusek and Lutheer, 1950; Reigart et al., 1975; Trabes et al., 1983). Death usually occurs in 3 h to 5 days from heart block, arrhythmia, or respiratory failure (Reigart et al., 1975). Important diagnostic symptoms registered with ECG are arrhythmia, the QT and ST intervals, and the T wave (Pattison, 1959; Taitelman et al., 1983b; Chi et al., 1996). The kidneys are among the most sensitive organs: acute renal failure associated with uremia and increased level of creatinine in serum can be observed under acute FA poisoning (Chung, 1984; Chi et al., 1996). Pathomorphological abnormalities of humans poisoned with FA are also nonspecific and similar to those of animals. In the case of lethal outcome, petechial hemorrhages and excess blood filling of internal organs (Hayes, 1982), edema of lungs and brain, and sometimes mediastinal emphysema and acute inflammatory reaction with coagulating necrosis in the esophagus were registered in humans (Brockmann et al., 1955). The morphological basis of cardiotoxic effects is acute myocardial dystrophy, a characteristic of which is diffuse lesions of cardiac muscle (Pattison, 1959; Taitelman et al., 1983b). Acute renal failure develops due to the influence of FA on subcellular structures of the kidneys. Metabolic acidosis aggravates the clinical course of renal failure. Diffuse degeneration of renal tubules was observed (Hayes, 1982). For cases that lack clinical and morphological specificity, biochemical data and primarily citrate and fluoride levels can be used for diagnostic purposes (Pattison, 1959; Schultz et al., 1982). Thus, under acute intoxication with FAA, citrate (108 μg/g in heart and 23.9 μg/g in kidneys) and fluoride (6.3 mg/g dry weight of heart and kidneys) were found in a human corpse; the dose of FAA was estimated to be near 23 mg/kg (Hayes, 1975). In addition, the indubitable diagnostic confirmation of the intoxication should be based on determination of the poison in tissues. Under acute SFA poisoning with lethal outcome, FA was found in urine (368 μg/mL), liver (58 μg/g), and brain (76 μg/g) (Harrison et al., 1952).

Among the aftereffects that develop after acute intoxication with FA or its derivatives are various neurological disturbances: impaired muscular tonus and reflex activity, transient spasmodic and meningeal syndromes, cerebellar dysfunction, such as ataxic gait, dysarthria, and intention tremor (Pridmore, 1978; Trabes et al., 1983; Kim and Jeon, 2009). Long after an acute poisoning (from 1.5 to 9 years) tendencies for epileptoid seizures, ataxia, extremity muscular hypertension, spastic tetraplegia, blindness

of cortical origin, diffuse brain atrophy, and psychic disorders were observed. A case of chronic intoxication with FA of a farm worker has been described (Parkin et al., 1977); the clinical signs were renal insufficiency and less pronounced injuries of other organs.

15.6 Treatment

Decades of studies on the toxicology of FA have led scientists to the conclusion that the treatment of intoxications can be successful only if timely general and symptomatic therapy is applied, but not specific antidotes (Dorman, 1990; Norris, 2001). Much experimental work over an extensive period has been undertaken in an effort to find effective donors of acetate groups, because of their property to inhibit conversion of FA to FC. Ethanol, monoacetin (glycerol monoacetate), acetamide, and cortisone acetate were tested for their potency to serve as antidotes (Hutchens et al., 1949; Chenoweth, 1949; Cole et al., 1955; Giller, 1956; Egyed, 1971; Egyed and Shlosberg, 1977). A therapeutic effect was revealed for simultaneous introduction of ethanol and acetate (Hutchens et al., 1949; Tourtelotte and Coon, 1949). Negative effects of monoacetin and acetamide were enhancement of hyperglycemia and metabolic acidosis, damage to capillaries and hemolysis of red blood cells, and an increase in the citrate concentration in different organs (Engel et al., 1954; Egyed and Shlosberg, 1973). The administration of cortisone acetate inhibited FC synthesis and prevented the development of ketosis, though there was increased hyperglycemia (Cole et al., 1955).

Several antidotes were tested for their capacity to activate transport of the TCA cycle intermediates through mitochondrial membranes. For this purpose, fluoromalate was proposed, though positive results were negligible (Peters, 1972). Malate was also tested, but proved to be effective only in in vitro experiments (Buffa et al., 1972). Also in vitro, glutathione and a series of SH-containing compounds (cysteamine and *N*-acetylcysteine) were tested (Mead et al., 1985). However, they were incapable of replacing glutathione in enzymatic defluorination of FA and have not found practical application. TCA cycle intermediates (succinate, malate, citrate, and glutamate) were tested, but did not exhibit a protective effect (Hutchens et al., 1949). A positive result was observed in experiments with mice, which were administered calcium gluconate and succinate (Omara and Sisodia, 1990). This therapy was barely more effective than ethanol. Some 16 years later another research group tested the therapy with cats, which are known to be much more sensitive to FA. Again, differences in survival between treated and nontreated animals were not significant ($P > .05$) (Collicchio-Zuanaze et al., 2006). Administration of calcium chloride to cats under acute intoxication with FA made it possible to postpone their deaths by up to 166 min; combination of calcium chloride with monoacetin gave a similar effect (Taitelman et al., 1983a). Nevertheless, calcium chloride caused a reduction in the QT interval and favored survival of humans in cases of their intoxication with FAA (Taitelman et al., 1983b).

Our strategy for the development of therapeutic means of treating acute FA intoxication was based on a deep analysis of the biochemical literature, together with our own experimental data. Thus, high sensitivity of aconitase to inhibition by superoxide anion and nitric oxide (Gardner et al., 1994; Andersson et al., 1998; Castro et al., 1998) means that ROS and NO could be competitive antagonists of FC to avert its effect on aconitase. Also, as considered earlier, during FA intoxication glutamate could be utilized in the TCA cycle through GDH or transaminases (Yu et al., 1976; Liang, 1977; Hassel et al., 1994). Moreover, the effects of FC could be prevented by prior introduction of isocitrate (bypass of inhibited aconitase) and fructose-1,6-bisphosphate (energy substrate for neurons) (Lian and Stringer, 2004).

We have demonstrated that FA can adversely affect mitochondrial functions only if pyruvate was available as a respiratory substrate, and that changes to the redox state of pyridine nucleotides (PN) or their leakage from MCh could be critical factors that impair mitochondrial respiration and lead to cell death (Zinchenko et al., 2007). Opening of the mitochondrial pore is a reversible phenomenon: prevention of oxidation and/or leakage of NAD(P)H from MCh can restore the normal functional state of MCh. For example, when succinate or glutamate was used as the respiratory substrate, mitochondrial functions were not affected by FA (Fig. 15.3A).

As for other alternative substrates, we suggest that the accumulating intracellular citrate could be one of them. As discussed earlier, blockade of citrate transport from MCh under FA intoxication is not an obligatory event, and citrate can enter cytosol to be further utilized by cICDH (Max and Purvis, 1965; Buffa et al., 1972). The cICDH activity is almost equally distributed between cytosol and MCh of astroglia and microglia, whereas cICDH accounts for about 75% of activity in neurons and oligodendrocytes (Minich et al., 2003). We have not found data on the ratio of mitochondrial and cytoplasmic aconitases in the cells of the nervous system, but it is interesting to note that a similar ratio of m- and cICDH exists in hepatocytes (Rakhmanova and Popova, 2006), and that c-aconitase accounts for 65% of the aconitase in these cells (Konstantinova and Russanov, 1996). In rat heart a similar ratio of m- and c-aconitases has been revealed: 35% and 65%, correspondingly (Medvedeva et al., 2002). Based on these data, one may suggest that an effective pathway for citrate utilization and NADPH synthesis exists in these (and other) cells in the case of

inhibition of m-aconitase. This alternative pathway could play a positive physiological role because NADPH might be used for anabolic reactions and heat generation, glutathione reduction and NO synthesis, and for regulation of blood vessel tone by means of ROS generation (Winkler et al., 1986; Bobyleva et al., 1993; Lee and Yu, 2002; Gupte and Wolin, 2006). As was pointed out earlier, studies focused on the pentose cycle as the main source of NADPH need to be re-evaluated taking into consideration the metabolic activity and substrate specificity of a tissue (Winkler et al., 1986). cICDH along with malic enzyme and transhydrogenase participates in NADPH regeneration to further reduce glutathione in brain mitochondria (Vogel et al., 1999), but cICDH can provide a sevenfold greater generation of NADPH as compared to malic enzyme (Winkler et al., 1986). The level of cytoplasmic NADPH can influence potassium channels and calcium balance (Wolin et al., 2005; Gupte and Wolin, 2006). In our in vitro studies, FA induced a slow elevation of $[Ca^{2+}]_i$ in different cells (Zinchenko et al., 2007). This could indicate an activation of the SOC channels; the process is not affected by FA and does need ATP to be implemented, at least in glial cells (Lian and Stringer, 2004). We suppose this mechanism to be common for many types of cells, and this could explain a primary hypersensitivity of platelets exposed to FA (Mindukshev et al., 2006). In cardiomyocytes, elevated $[Ca^{2+}]_i$ can stimulate their functional activity observed in our experiments in vitro and also supported in vivo by a primary increase in the systolic index (Table 15.1). As for modulating effects of Ca^{2+} on bioenergetics of MCh, it is pertinent to recall "classic" activation of the TCA cycle dehydrogenases followed by increase of mitochondrial potential and NADH generation: 2-oxoglutarate dehydrogenase (OGDH) and mICDH can be activated by calcium ions through allosteric mechanisms and pyruvate dehydrogenase is activated due to dephosphorylation by the Ca^{2+}-dependent phosphatase (McCormack et al., 1990; Hansford, 1994). The exact role of these dehydrogenases in the bioenergetic status of MCh affected by FA needs to be clarified, though one can suppose that OGDH could derive a special benefit from such an activation if it is provided with exogenic or endogenic 2-oxoglutarate.

According to the above discussion, we have defined several directions for biochemical correction under acute intoxication with FA and suggested suitable preparations for therapeutic complexes: (1) competitive inhibition of FA and CoA interaction; (2) competitive inhibition of FC and aconitase interaction; (3) replenishment of the TCA cycle distally of aconitase; and (4) utilization of accumulating citrate. In previous publications we presented the first data on the effectiveness of therapeutic complexes named METIS (Goncharov et al., 2006, 2009). These consisted of ethanol, methylene blue (MB), sodium glutamate (SG), and glycerol trinitrate (GT). Indices of therapeutic efficiency (ratio $LD_{50}^{treated}/LD_{50}^{nontreated}$) for different METIS complexes applied in different regimens are presented in Table 15.4. In addition to these data, a spectrum of physiological and biochemical data was also obtained. Animals treated with METIS complex had little changes to body weight, temperature, and oxygen consumption. The dynamics of citrate in brain, kidneys, and blood was also improved, and kinetic parameters of platelet aggregation were corrected. Comparative analysis of the FA level

TABLE 15.4 Assessment of the therapeutic effectiveness of METIS preparations under acute intoxication of rats with SFA.

Therapy	Treatment regimen after the poisoning with SFA	Index of therapeutic efficiency
		Ratio LD_{50} treated/LD_{50} nontreated
Ethanol ($n = 42$)	10 and 120 min	1.6
METIS-1 ($n = 48$)	10 and 120 min	2.5
METIS-2 ($n = 92$)	10, 60 and 120 min (ethanol and GT); 60 and 120 min (MB)	3.3
METIS-4 ($n = 39$)	10, 60 and 120 min, 24 h and 48 h (ethanol, GT, and SG); 10 and 120 min, 24 h and 48 h (MB)	4.3
Ethanol ($n = 43$)	30 and 120 min	2.6
METIS-3 ($n = 45$)	30 and 120 min	3.2

n, Number of animals used in the experiment to calculate the index.
Experimental conditions and METIS components. SFA was dissolved in distilled water and administered intragastrically at 0.2 mL per 100 g of rat body weight, after 7–8 h fasting. METIS-1 is a combination of two compounds: aqueous solution of MB administered subcutaneously (s.c.) at a dose of 5 mg/kg, and aqueous solution of SG administered intraperitoneally (i.p.) at a dose of 250 mg/kg. METIS-2 is a combination of three compounds: MB (5 mg/kg, s.c.), ethanol and GT administered i.p. (corresponding doses for pure ethanol and GT were 1 g/kg and 10 mg/kg). METIS-4 is a combination of four compounds: MB (5 mg/kg, s.c.), and aqueous solution of ethanol (1 g/kg), GT (10 mg/kg), and SG (100 mg/kg) administered i.p. METIS-3 is a combination of three compounds: MB (5 mg/kg, s.c.), ethanol, and SG administered i.p. (corresponding doses for pure ethanol and SG were 1 g/kg and 200 mg/kg).

in tissue homogenates, blood plasma, and urea of rats revealed that the METIS complexes reduced the level of FA in the brain almost twofold, thus indicating inhibition of FA utilization first of all in the cells of the nervous system.

15.7 Concluding remarks and future directions

The extreme toxicity of FA is determined by its similarity to acetate, which has a central role in cell metabolism. FA enzymatically condenses with CoA-SH to produce fluoroacetyl-CoA, which replaces acetyl-CoA entering the TCA cycle and produces FC. The latter reacts with aconitase and blocks the TCA cycle. Energy production is reduced, as well as the concentration of metabolites generated distally to aconitase. 2-Oxoglutarate is the most important of these, being a precursor of glutamate, which is a neuromediator in the CNS and participates in neutralizing ammonia either directly through glutamine synthase or indirectly through the urea cycle. Accumulation of citrate is one of the causes of metabolic acidosis. Chelating of Ca^{2+} is apparently one of the central events in the pathogenesis of intoxication.

The first papers on FA toxicology were published in the 1940s. The long history of investigations was fruitful, with several important discoveries: biochemical mechanism of "lethal synthesis"; structure of aconitase; functional relations of glia and neurons; and switching of metabolic pathways. However, the main problem of toxicology (for any poison) was not solved—development of an effective therapy. Analysis of the scientific literature has demonstrated that reciprocal relations of signaling and metabolic pathways under intoxication with FA are unclear. Inhibition of m-aconitase causes blockade of the TCA cycle, reduction of pyridine nucleotides, accumulation of citrate, disturbances of intracellular signaling, deenergization, and cell death. However, the dynamics and significance of these events are different depending on the type of cells and tissues, which is why it is very difficult to predict the primary reaction of different cells and more so the whole organism.

Biochemical pathways underlie the basis of physiological rhythms; they should have a certain space−time structure and presuppose coordinated interactions of different cells. Thus, one of the causes of disturbances of normal respiration under FA intoxication could be disturbances in rhythmic activity of respiratory neurons; but suppression of these neurons is a consequence of an inhibiting effect of FA on astrocytes, not neurons (Hulsmann et al., 2000). We described the development of cardiac and respiratory tachyarrhythmias reflecting reproduction of decasecond rhythms characteristic of immature or abnormal excitatory structures (Kuznetsov et al., 2007). Previously it was suggested that such endogenic rhythmic activity could be determined by the level of pentose cycle activity (Kuznetsov, 2002). This cycle plays an important role in neurons, protecting them from oxidative or traumatic stress (Ben-Yoseph et al., 1994; García-Nogales et al., 2003; Bartnik et al., 2005). However, it should be noted that although the activity of NADPH-generating enzymes of the pentose cycle in astrocytes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) is two- to threefold higher than in brain as a whole, the activity of cICDH is also very high in these cells (Rust et al., 1991). This metabolic pathway is interesting primarily because under FA intoxication citrate is accumulated, and the pathway may be regarded as a form of biochemical adaptation that facilitates utilization of the central metabolite. It was suggested that spatial and temporal division of m- and c-aconitases do not only provide regulation of iron balance in cells, but also provide regulation of balance between catabolic and anabolic processes (Tong and Rouault, 2007).

Providing the cells have utilized citrate entering the cytosol, another problem should be utilization of generating NADPH. One of the possible and very important mechanisms of PN oxidation is heat generation through shiver and nonshivering thermogenesis. A rise in the activity of NADPH-generating enzymes and pathways, including c-aconitase and cICDH, is accompanied by enhanced thermogenesis (Bobyleva et al., 1993). It was shown that NADPH could be used together with or even instead of NADH as a reducing cofactor for cytoplasmic glycerophosphate dehydrogenase (Bobyleva et al., 1993; Fahien et al., 1999). However, if the role of this pathway for transferring electrons from cytoplasma to MCh in skeletal muscles is rather clear, then the level of activity and functional state of glycerophosphate shuttle in brain cells are contradictive. For example, its activity in brain is explained by the need for glycerol-3-phosphate as a substrate for phospholipid synthesis in oligodendroglia (Adler and Klucznik, 1982; Nguyen et al., 2003). In neurons and astrocytes, the activity of glycerophosphate dehydrogenases is much lower than in oligodendrocytes (Rust et al., 1991; Nguyen et al., 2003). There are data, however, that indicate an important role of this shuttle in astrocytes, taking into consideration: (1) the absence of malate−aspartate shuttle in these cells (Waagepetersen et al., 2001; McKenna et al., 2006), and (2) the elevated level of mRNA of cICDH in astrocytes after convulsions, under exposure to morphine, indometacin, and some other preparations (Link et al., 2000).

It is noted that FA can prevent the development of tolerance to morphine (Song and Zhao, 2001). Another positive aspect of FA in nontoxic concentrations is its radioprotective power due to its capacity to reduce body

temperature and oxygen consumption (Misustova et al., 1980). Moreover, we have estimated that SFA significantly inhibits growth of Ehrlich tumor carcinoma. In experiments with autochthonous induced by benzo[a]pyrene subcutaneous tumors, SFA was not active in the monotherapy regimen, though it exhibited enhancement of the antitumor effect of cyclophosphamide, significantly increasing the number of mice with stabilized or decreased tumor volume as well as the duration of this effect (Anikin et al., 2013). These findings provide a basis for additional studies of the mechanism of the antitumor effect of SFA. Thus, we suggest that future progress in toxicological (and pharmacological) studies of FA will depend on comprehensive consideration of these and more recent data, together with re-evaluation of old and forgotten data.

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Chapter 16

Strychnine

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16.1 Introduction

Strychnine is a poisonous indole-type alkaloid found in the genus *Strychnos*. Its basic compound forms colorless or white rhombic crystals. These have a bitter taste and melt at approximately 290°C. Strychnine was the first alkaloid to be identified in plants of the genus *Strychnos*, Family Loganiaceae. *Strychnos*, created by Linnaeus in 1753, is a genus of trees and climbing shrubs of the gentian order. The genus contains 196 various species and is distributed throughout the warm regions of Asia (58 species), America (64 species), and Africa (75 species). Plants of the genus *Strychnos* have opposite leaves and bear cymes of white or yellowish flowers that have a four-lobed or five-lobed calyx, a four-part or five-part corolla, five stamens, a solitary pistil, and they bear fruit in the form of a berry. The seeds and bark of many plants in this genus contain the powerful poison strychnine. Strychnine is obtained commercially from the seeds of the Saint Ignatius bean (*Strychnos ignatii*) and from the nux-vomica tree (*Strychnos nux-vomica*) (Volfova and Patocka, 2003). *S. nux-vomica*, also known as poison nut, semen strychnos, and quaker buttons, is a deciduous tree native to India and southeast Asia. It is a medium-sized tree that grows in open habitats. The seeds contain approximately 1.5% strychnine and the dried blossoms contain 1.0% (Harry, 1968). However, the tree's bark also contains other poisonous compounds (Guo et al., 2018). Strychnine was first discovered in the Saint Ignatius bean by French chemists Joseph-Bienaime Caenoiu and Pierre-Joseph Pelletier in 1818. In some *Strychnos* plants the 9,10-dimethoxy derivative of strychnine brucine, is also present (Li et al., 2006). Strychnine, along with its close relative brucine, has been found in a number of trees growing in the tropical regions of the world, especially in India and the Far East. Brucine is not as poisonous as strychnine (Teske et al., 2011).

16.2 Background

16.2.1 Chemistry and physicochemical properties

Strychnine has the molecular formula $C_{21}H_{22}N_2O_2$, and its structural formula is shown in Fig. 16.1 [CAS No: 57-24-9 (base), 60-41-3 (sulfate)]. It occurs as white crystals or powder that is odorless, with a melting point of 286°C, boiling point of 270°C at 5 mmHg, density of 1.36 g/cm^3, vapor density of 11.0 (air = 1), and vapor pressure 0 torr at 20°C (Mackison et al., 1981). Strychnine is a stable compound and incompatible only with strong oxidizing agents.

Water solubility of strychnine is negligible, only 160 mg/L at 25°C and pH of the saturated solution is 9.5. Octanol/water partition coefficient ($\log K_{ow}$) is 1.93 (Hansch et al., 1995). Strychnine is very slightly soluble in ether, slightly soluble in benzene, ethanol (6.7 g/cm^3), and acetone, and soluble in chloroform and pyridine (Ng and Poe, 1956; Budavari, 1996).

16.2.2 History

The toxic and medicinal effects of strychnine have been well-known since the times of ancient China and India. The inhabitants of these countries had ancestral knowledge of the species *nux vomica* and Saint Ignatius bean. The species *S. nux vomica* is a tree of native Indonesia that attains a height of 12 m. The tree has a crooked, short, thick trunk and the wood is close-grained and very durable. The fruit has an orange color and is approximately the size of a large apple with a hard rind and contains five seeds that are covered with a soft wool-like substance. The ripe seed looks like flattened disks and is very hard. These seeds are the chief commercial source of strychnine and were first imported and marketed to

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FIGURE 16.1 Chemical structure of strychnine.

Europe as a poison to kill rodents and small predators. *S. ignatii* is a woody climbing shrub of the Philippines. The fruit of the plant contains as many as 25 seeds embedded in the pulp. The seeds contain more strychnine than other commercial alkaloid plants. The properties of *nux-vomica* and Saint Ignatius seeds are substantially those of the alkaloid strychnine (Haller, 1973; Buckingham, 2007).

Strychnine was discovered and identified as the main toxic principle of *Strychnos* in 1818, although *nux vomica*, the unpurified plant extract in which it is the active component, had been known and used for both medicinal and criminal purposes for some time. Historic records indicate that the strychnine alkaloid has been used to kill dogs, cats, and birds in Europe as far back as 1640. The structure of strychnine was first determined in 1946 by Sir Robert Robinson and in 1954 this alkaloid was synthesized in the laboratory by Robert W. Woodward (Woodward et al., 1954). This is one of the most famous syntheses in the history of organic chemistry. Both chemists won the Nobel prize, Robinson in 1947 and Woodward in 1965.

16.2.3 Therapeutic uses

Strychnine does have some history of use for therapeutic purposes, although in most cases this was entirely misguided and dangerous. It has a very bitter taste and so stimulates salivary and gastric secretion. This increases appetite and was used to counteract the loss of appetite associated with illness, giving the impression that strychnine had restorative properties. In fact, any constitutional improvement resulting from the increased appetite would probably be outweighed by the harmful effects of strychnine, and the underlying illness would be more or less unaffected. This is the same mechanism that gives tonic water its apparent "tonic activity," although in that case the bitter agent is quinine, which is much less dangerous (McGarry and McGarry, 1999).

Strychnine has an undeserved reputation as a useful therapeutic agent. There is no current justification for its presence in any medication. However, preliminary experimental reports suggest that judicious treatment with strychnine may modify the neurological deterioration in some infants with nonketonic hyperglycinemia, a rare metabolic disorder characterized by abnormally high concentrations of glycine in the brain and cerebrospinal fluid (MacDermot et al., 1980; Warburton et al., 1980). Strychnine was used as a tonic stimulant at one time for atonic constipation and as a stomachic and bitter. It produces no selective gastrointestinal effects and has no place in the therapy of any gastrointestinal disorder. At present strychnine has no demonstrated therapeutic value in human medicine, despite a long history of unwarranted popularity. It was first used in medicine in 1540, but it did not gain wide usage until 200 years later (Gilman et al., 1985; Hur et al., 2019).

Although strychnine was previously used extensively in animals, it no longer has a rational place in the therapeutic resources of the veterinarian. Its only interest is from a veterinary toxicology standpoint because animals are sometimes poisoned inadvertently or intentionally (Booth and McDonald 1982; Motas-Guzmán et al., 2003; Berny et al., 2010; Cowan and Blakley, 2015, 2016; Gupta, 2018).

16.3 Pharmacokinetics and toxicokinetics

16.3.1 Absorption, distribution, metabolism, and excretion

Strychnine is rapidly absorbed from the gastrointestinal tract, mucous membranes, and parenteral sites of injection (Thienes and Haley, 1972) as well as from the oral cavity (LaDu et al., 1971). A nonfatal case of strychnine poisoning through dermal exposure has been described (Greene and Meatherall, 2001). Strychnine is transported by plasma and erythrocytes, but protein binding is slight and distribution to the tissues occurs rapidly. Within a few minutes of ingestion strychnine can be detected in the urine and excretion via that route accounts for approximately 15% of a sublethal (4 mg) dose over 6 h. Little difference was noted between oral and intramuscular administration of strychnine in a 4 mg dose. Blood levels in human poisoning were less than 0.5 μg/mL from 1 to 48 h after ingestion of a sublethal dose (700 mg), 2.7 μg/mL in a patient who survived the acute episode, and 40 μg/mL in a patient who died after massive ingestion (Haddad and Winchester, 1983). In persons killed by strychnine, the highest concentrations are found in the blood, liver, and kidney (Hayes and Laws, 1991). The half-life of absorption is approximately 15 min and the half-life of metabolism is approximately 10 h. The apparent volume of distribution in one patient was 13 L/kg (Ellenhorn et al., 1997).

Strychnine is rapidly metabolized by the liver microsomal enzyme system and requires NADPH and O_2. Five metabolites formed in vitro by rabbit liver were isolated and identified as 2-hydroxystrychnine, 11,12-dehydrostrychnine, strychnine-21,22-epoxide, 21,22-dihydroxy-22-hydrostrychnine, and strychnine-*N*-oxide, which was the major metabolite and accounted for approximately 15% of the metabolized strychnine. All other metabolites accounted for less than 1% (Mishima et al., 1985). Similar metabolites were identified in rat urine where the major metabolite was strychnine-21,22-epoxide (Oguri et al., 1989). The metabolic fate of strychnine in humans is not known.

Strychnine competes with the inhibitory neurotransmitter glycine, producing an excitatory state characterized clinically by hyperreflexia, severe muscle spasms, and convulsions. However, the toxicokinetics after overdose have not been well-described. In most severe cases of strychnine poisoning, the patient dies before reaching hospital (Shadnia et al., 2004; Prat et al., 2015).

Palatnick et al. (1997) described the case of a 34-year-old man who presented to the emergency department 20 min after ingesting 125 mL of 2% strychnine sulfate (2.25 g). He was alert and oriented and experiencing muscle spasms. His condition deteriorated, prompting sedation, muscle paralysis, and tracheal intubation. He was administered activated charcoal 100 g via nasogastric tube. He was admitted to intensive care, where he was managed with diazepam, pentobarbital, and pancuronium. Despite mild rhabdomyolysis, he recovered and was extubated on day 3. Although receiving prophylactic heparin therapy, a massive fatal pulmonary embolus ensued. Eighteen blood specimens for strychnine analysis were obtained from 20 min to 51 h after ingestion. Serum concentrations were determined with gas chromatography-mass spectroscopy. Disappearance followed a first-order process with a $t_{0.5}$ of 16 h. These results confirm the findings of a previous case report of 19 strychnine levels obtained between 4 and 19 h that described first-order kinetics with a $t_{0.5}$ of 10−16 h.

In a case report by Wood et al. (2002), a 42-year-old man ingested an unknown quantity of strychnine powder. Eight serum samples were taken over the first 5 days and analyzed subsequently for strychnine concentrations. The initial concentration at 1.5 h after ingestion was 4.73 mg/L, falling to 0.38 mg/L at 74 h postingestion. Serum concentrations followed a monoexponential elimination curve with a calculated elimination half-life of 12 h. The initial serum concentration of 4.73 mg/L is the highest reported concentration in a patient who has survived.

16.4 Clinical symptomatology

Symptomatology of human intoxication begins 15−30 min after ingestion of strychnine, usually without any warning, and the subject may experience violent convulsions. Convulsions lead to severe lactic acidosis that secondarily results in visceral (lung, heart, kidney, liver, and brain) collapse and death (Gordon and Richards, 1979). Prodromal symptoms are described such as apprehension, restlessness, heightened acuity of hearing, vision, and feeling, hyperreflexia, abrupt movement, and muscular stiffness of the face and legs. Generalized convulsions last from 30 s to 2 min. At 10−20 min after exposure, the body's muscles begin to spasm, starting with the head and neck. At first convulsions are clonic, but a tetanic phase quickly follows. The body typically arches in hyperextension, the legs are adducted and extended, arms are flexed over the chest and fists are tightly clenched. The jaw is rigidly clamped, the face is fixed in a grin, and the eyes protrude in a fixed stare (Philippe et al., 2004; Lages et al., 2013).

The convulsions progress, increasing in intensity and frequency until the backbone arches continually. Death comes from asphyxiation caused by paralysis of the neural pathways that control breathing or by exhaustion from the convulsions. The subject usually dies within 2−3 h after exposure. At the point of death, the body "freezes" immediately, even in the middle of a convulsion, resulting in instantaneous rigor mortis.

Initial symptoms of strychnine poisoning are tightness and twitching of the muscles, agitation, and hyperreflexia. Stiffness of the body, lockjaw, frothing of the mouth, and cessation of respiration occur. Tetanus-like episodes occur every 10−15 min. During these episodes, the eyeballs protrude and the pupils enlarge. Severe cyanosis, which disappears after the episode subsides, also occurs. The episodes (each lasting approximately 3−4 min) appear to be spontaneous, while other times they are the result of external stimuli, for example, noises, slight movements, or flashes of light. The patient never loses consciousness. When the poisoning is left untreated, each episode lasts longer than the previous and the interval between them grows shorter. Up to 10 episodes occur before death or recovery. This could happen from 10 min to 3 h and is a result of asphyxiation or inner tissue paralysis.

16.5 Mechanism of action

Strychnine acts as a blocker or antagonist at the inhibitory or strychnine-sensitive glycine receptor, a ligand-gated chloride channel in the spinal cord and the brain (Song et al., 2006). The glycine receptor (GlyR) is the receptor for the amino acid neurotransmitter glycine (Rajendra et al., 1997). It is one of the most widely distributed

inhibitory receptors in the central nervous system. Glycine receptors are found in most brain areas, including the hippocampus, amygdala, ventral tegmental area, and periaqueductal gray (Choi et al., 2013). The strychnine-sensitive glycine receptor is a member of a family of ligand-gated ion channels (Alexander et al., 2015). This ionotropic receptor can be activated by a range of simple amino acids, except glycine, β-alanine, and taurine, and can be selectively blocked by the high-affinity competitive antagonist strychnine. The receptor is arranged as five subunits surrounding a central core, with each subunit composed of four α-helical transmembrane segments. There are presently four known isoforms of the α-subunit (α_{1-4}) of GlyR that are essential to bind ligands and a single β-subunit (Huang et al., 2015). The adult form of the glycine receptor is the heteromeric $\alpha_1\beta$ receptor, which is believed to have a stoichiometry of three α_1 subunits and two β subunits or four α_1 subunits and one β subunit (Kuhse et al., 1993, 1995). The strychnine-binding subunit of the glycine receptor shows certain homology with nicotinic acetylcholine receptors (Grenningloh et al., 1987). The glycine receptor is highly enriched in microdomains of the postsynaptic neuronal surface apposed to glycinergic afferent endings. There is substantial evidence suggesting that the selective clustering of the glycine receptor at these sites is mediated by the cytoplasmic protein gephyrin (Meier et al., 2000). Gephyrin is a multimeric scaffold protein which interacts with cytoskeletal elements and stabilizes GlyRs and individual subtypes of gamma-aminobutyric acid A receptors at inhibitory postsynaptic sites (Maas et al., 2006).

16.6 Toxicity

Strychnine has been placed in toxicity category I, indicating a high degree of acute toxicity, for oral and ocular effects. Inhalation toxicity is also presumed to be high. Acute toxicity of strychnine to birds is very high. Mammalian studies indicate that strychnine is very highly toxic to small mammals on both an acute oral basis and a dietary basis. The extent of poisoning caused by strychnine depends on the amount and route of strychnine exposure; in humans, it depends on the person's condition of health. The signs of toxicity, including death, occur within 1 h.

16.6.1 Animal toxicity

Reported toxic doses of strychnine administered by different routes in some animals and humans are summarized in Table 16.1.

Strychnine toxicity in rats depends on sex. It is more toxic to females than to males when administered subcutaneously or intraperitoneally, and differences are attributable to the higher rate of metabolism by male rat liver microsomes (Parke, 1968). Dogs and cats are more susceptible among the domestic animals, pigs are believed to be as susceptible as dogs, and horses are able to tolerate relatively larger amounts of strychnine (Humphreys, 1988). Birds affected by strychnine poisoning exhibit feathers fluffed or held tightly against the body, ataxia, wing droop, salivation, tremors, muscle tenseness, and convulsions. Death occurs as a result of respiratory arrest.

The clinical signs of strychnine poisoning relate to its effects on the central nervous system. After oral ingestion of strychnine, symptoms of poisoning usually appear within 15−60 min. The first clinical signs of poisoning include nervousness, restlessness, twitching of the muscles, and stiffness of the neck. As the poisoning progresses, muscular twitching becomes more pronounced and convulsions suddenly appear in all the skeletal muscles. The limbs are extended and the neck is curved to opisthotonus. The pupils are widely dilated. As death approaches, the convulsions follow one another with increased rapidity, severity, and duration. Death results from asphyxia because of prolonged paralysis of the respiratory muscles (Humphreys, 1988; Gupta, 2018).

16.6.2 Human toxicity

People exposed to low or moderate doses of strychnine by any route will have the following signs or symptoms: agitation; apprehension or fear; ability to be easily startled; restlessness; painful muscle spasms possibly leading to fever and to kidney and liver failure; uncontrollable arching of the neck and back; rigid arms and legs; jaw tightness; muscle pain and soreness; difficulty breathing; dark urine; and initial consciousness and awareness of symptoms. The reported medium lethal doses of strychnine in humans ranges from 5 to 120 mg/kg.

Cases of human poisoning have been reported. A 46-year-old man presented 2 h after ingestion of approximately 250 mg strychnine with severe, violent, generalized convulsions triggered by external stimuli. During the convulsion-free periods, there were no abnormal signs in the physical examination (Scheffold et al., 2004). A 28-year-old man was admitted 2 h after ingestion of 1−1.5 g of strychnine. He was severely agitated and in mild respiratory distress; blood pressure was 90/60 mmHg, pulse was 110 per minute, and peripheral pulses were weak. He had generalized hyperactive reflexes and had several generalized tonic-clonic convulsions in the emergency department. Treatment consisted of gastric lavage with water, oral administration of activated charcoal and sorbitol solution, continuous intravenous administration of midazolam, followed by sodium thiopental, furosemide, sodium bicarbonate, and hemodialysis for acute renal failure. His clinical course included respiratory distress,

TABLE 16.1 Toxicity values (LD_{50}) of strychnine found in different literature sources.

Organism	Route	LD_{50} (mg/kg)	Source
Bird—wild	Oral	16	Tucker and Haegele (1971)
Cat	Intravenous	0.33	RTECS (1935)
Cat	Oral	0.5	Moraillon and Pinault (1978)
Dog	Intravenous	0.8	Longo et al. (1959)
Dog	Subcutaneous	0.35	RTECS (1935)
Dog	Oral	0.5	Moraillon and Pinault (1978)
Duck	Oral	3.0	Tucker and Haegele (1971)
Human	Oral	100–120	Zenz et al. (1994)
Human (adult)	Oral	30–100	Gossel and Bricker (1994)
Human (children)	Oral	15	Gossel and Bricker (1994)
Human	Oral	30–60	Lewis (1996)
Human	Oral	5–10	Ellenhorn et al. (1997)
Human (adult)	Oral	50–100	Migliaccio et al. (1990)
Human	Oral	100–120	Palatnick et al. (1997)
Mouse	Intraperitoneal	0.98	Setnikar et al. (1960)
Mouse	Intravenous	0.41	Haas (1960)
Mouse	Oral	2.0	Prasad et al. (1981)
Mouse	Parenteral	1.06	Zapata-Ortiz et al. (1961)
Mouse	Subcutaneous	0.47	Sandberg and Kristianson (1970)
Pigeon	Oral	21.0	Tucker and Haegele (1971)
Quail	Oral	23.0	Tucker and Haegele (1971)
Rabbit	Intravenously	0.4	Longo et al. (1959)
Rabbit	Oral	0.6	RTECS (1935)
Rat	Oral	16.0	Spector (1956)
Rat	Oral	2.35	Ward and Crabtree (1942)

RTECS, Registry of Toxic Effects of Chemical Substances.

agitation, generalized tonic-clonic convulsions, hyperactivity, oliguria, and acute tubular necrosis before recovery in 23 days. This patient ingested what would normally be a fatal amount of strychnine. He had signs and symptoms of severe toxicity and recovered, suggesting that with aggressive supportive care patients may have favorable outcomes (Shadnia et al., 2004). In another case report described by Wood et al. (2002), a 42-year-old man presented soon after ingestion of an unknown but warranted lethal quantity of strychnine powder. After respiratory arrest with intensive supportive management requiring admission to an intensive care unit, he survived.

People exposed to high doses of strychnine may have respiratory failure, possibly leading to death or brain death, within the first 15–30 min of exposure. No postmortem lesions are observed with the exception of small pinpoint hemorrhages in the lungs resulting from death attributable to asphyxia. Rigor mortis occurs soon after death and persists for days.

Toxicity of strychnine in humans, expressed as LD_{Lo} (lethal dose low), is approximately 30 mg/kg. Strychnine is less toxic in humans than in most animals. If the person survives the toxic effects of strychnine poisoning, long-term health effects are unlikely. However, long-term effects may result from damage caused by the poisoning, for example, brain damage from low oxygen or kidney failure. People severely affected by strychnine poisoning are not likely to survive.

At the present time, fatal strychnine poisoning is uncommon. It is no longer used as a therapeutic drug and its availability to the public is controlled by legislations in various jurisdictions. Nevertheless, it is still in use as a rodenticide, a herbal remedy, and an adulterant in street drugs (Cole et al., 2011; Singhapricha and Pomerleau, 2017). Illicit drugs very often contain substances that are a serious risk to health. Strychnine may be one such substance (Prat et al., 2015). Homicide by strychnine is extremely rare (Lynch, 1948; Bogan et al., 1966; Jaulmes and Hamelle, 1968). Autopsy findings are subtle, so strychnine poisoning could easily be overlooked and a homicide might go undetected. It is important in deaths in which there are no gross autopsy findings, sudden death in particular, that routine toxicology be performed because strychnine is likely to be detected (Kodikara, 2012). Exceptionally, strychnine has been used as a poison to commit suicide (Kordrostami et al., 2017). In the Western world, strychnine poisoning is much less common.

16.6.3 Diagnosis

A tentative diagnosis can be made based on clinical signs and history. However, a positive diagnosis can only be made by identifying strychnine in the stomach contents, viscera, or blood. The drug can be identified by chemical tests and microscopic identification of typical strychnine crystals. Sensitive analytical methods have already been developed to identify and quantify strychnine in various biological materials (Egloff et al., 1982; Wang et al., 2004; Neely et al., 2018).

16.7 Risk assessment

16.7.1 Human health hazard

The human health assessment for strychnine is based on acute toxicity. Strychnine has been placed in Toxicity Category I, indicating the greatest degree of acute toxicity, for oral and ocular effects. It has been reported that the probable lethal oral dose is 1.5−2 mg/kg (Gosselin et al., 1984). Inhalation toxicity is also presumed to be high. An oral dose of 1.5−2 mg/kg is equivalent to 70−93 mg/m^3 for 30 min for a 70-kg human.

Strychnine was first registered as a pesticide in the United States in 1947; however, this natural toxin had been used in many counties to control vertebrate animals for many years before that time. Currently, strychnine is registered for use only below ground as a bait application to control pocket gophers. The end-use products are formulated as grain-based bait or pastes. Baiting can be performed manually or with the use of application equipment.

The European Union (EU) withdrawal of strychnine marked its end as a method of mole control. The EU directive 91/414/EEC is midway through an ambitious program to review all pesticides used within Member States. This requires manufacturers to provide health and safety data to support the continued registration of their products. Strychnine was to be reviewed in the fourth part of this program, but manufacturers have failed to provide such data. Despite last-ditch appeals by users, since September 1, 2006, strychnine has no longer been legal to use.

However, strychnine in the form of a homeopathic preparation is still used and in exceptional cases may also cause poisoning. Gicquel et al. (2012) presented the case of a bulimic woman who was admitted to the emergency unit for painful muscle spasms and hypertonic crisis with respiratory blocking, after application of homeopathic nux vomica mother tincture, which contains indole alkaloids including strychnine. Toxicological screening revealed the presence of strychnine in the blood sample. This was corroborated by the patient, who explained that she had swallowed a whole bottle of nux vomica mother tincture as an emetic during a bulimic episode. This corresponded to 212 mg strychnine ingested. The therapeutic patient management consisted of symptomatic medication by diazepam and paracetamol and monitoring of biological parameters and vital functions including respiratory functions. Determination of strychnine concentrations in blood samples contributed to the control of poisoning. A blood concentration of 3 mg/L was first evaluated at admission of the patient, followed by a progressive decrease to 0.5 mg/L on the second day and a favorable clinical outcome for the patient.

16.7.2 Safety data

Strychnine oral reference dose (RfD) of 0.0003 mg/kg/day or 0.02 mg/day for a 70-kg person is derived from the Seidl and Zbinden (1982) short-term to subchronic study by applying an uncertainty factor of 10,000. This factor accounts for extrapolation from a less-than-chronic to a chronic exposure study, extrapolation from animals to humans, and differences in sensitivity among the human population. An additional factor of 10 is used because a LOAEL/FEL (2.5 mg/kg/day) was utilized in the estimation of the RfD instead of a NOAEL. The immediate dangerous to life and health dose for strychnine by NIOSH REL is 0.15 mg/m^3, and the current OSHA PEL is 0.15 mg/m^3.

The work of Seidl and Zbinden (1982) is the only oral short-term or subchronic study reported in which rats received daily doses of 0−10 mg/kg of strychnine by gavage for 28 days. Data recorded for the surviving animals included blood cell counts, electrocardiograms, eye

examinations, urine chemistry, weight gain, tissue histology, organ weights, behavioral tests, and food and water consumption. Mortality was observed in 5 of 12 male rats receiving 10 mg/kg and in 1 of 12 in each of the 5 mg and 2.5 mg/kg groups. All deaths occurred 0.5−6 h after oral doses.

An additional study (Gitzelmann et al., 1978) reported that a 6-month-old human patient received strychnine doses of 0.3−1.1 mg/kg/day over an 18-month period without any adverse effects. However, the patient may have had a higher strychnine tolerance as a result of nonketotic hyperglycinemia. Risk phrases of strychnine: R27/R28 R50/R53.

16.8 Treatment

There is no specific antidote for strychnine, but recovery from strychnine exposure is possible with early hospital treatment. Treatment consists of removing the drug from the body (decontamination) and getting supportive medical care in a hospital setting. Supportive care includes intravenous fluids, medications for convulsions and spasms, and cooling measures for high temperature.

Treatment of strychnine poisoning involves an oral application of an activated charcoal infusion that serves to absorb any poison within the digestive tract that has not yet been absorbed into the blood. Unabsorbed strychnine can be removed from the stomach by gastric lavage of tannic acid (strong tea) or potassium permanganate solutions used as chemical antidotes. Seizures are controlled and anticonvulsants such as phenobarbital or diazepam are administered to control convulsions, along with muscle relaxants such as dantrolene to combat muscle rigidity. Diazepam is the anticonvulsant of choice but is not effective in all cases, so a combination with midazolam, fentanyl, and pancuronium is recommended for controlling convulsions (Scheffold et al., 2004). To avoid a fatal outcome of strychnine poisoning demands aggressive management with early intubation, control of muscle tremors, and prevention of rhabdomyolysis and renal failure. If the patient survives past 24 h, then recovery is probable.

Small doses of strychnine were once used in medications as a stimulant, a laxative, and as a treatment for other stomach ailments. Strychnine has stimulant effects at low doses, but because of its high toxicity and tendency to cause convulsions, the use of strychnine in medicine was eventually abandoned once safer alternatives became available.

16.9 Concluding remarks and future directions

Strychnine is a highly poisonous natural substance that is used in some countries for the control of wild animals. Today, strychnine is used primarily as a pesticide, particularly to kill rats. Its use is restricted by law. Because strychnine is highly toxic and can be rapidly absorbed through the mucous membranes of the mouth, stomach, and small intestines, theoretically it could be used as a military toxin or terrorist agent. There are three main ways that strychnine can enter the body: inhalation, ingestion, and broken skin.

Goal-directed misuse of strychnine against humans is unlikely. Its misuse against domestic animals is realistic and more likely.

Uncommonly, strychnine is found mixed with "street" drugs such as LSD, heroin, and cocaine. It is very probable that seizures observed occasionally after cocaine application may be caused by admixed strychnine (Haddad and Winchester, 1983; Wijesekera et al., 1988). After analysis of heroin samples seized in the Florence area between 1975 and the first half of 1981, no dangerous substances were found in the samples and strychnine, if present, was found in very low concentrations (Mari et al., 1982).

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Chapter 17

Superwarfarins

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17.1 Introduction

Chemical warfare agents may be manufactured from a wide range of commercially manufactured household industrial products, such as bleach, antifreeze, fertilizers containing anhydrous ammonia, pesticides, or anticoagulant rodenticides, particularly superwarfarins (Feinstein et al., 2016), Superwarfarins are a group of commercially available, long-acting, anticoagulant rodenticides that are structurally similar to warfarin but are many times more potent. Many of them have the capacity to cause severe bleeding problems that may last for 2−8 months in humans. Superwarfarins may be used to harm or terrorize people through the ingestion of contaminated food, water, or drugs.

This group of long-acting anticoagulants may be used as chemical warfare agents because of their high potency and duration of action. The capability of terrorists to use these commercially available poisons depends upon the availability of large amounts of high-concentration product (whether obtained legally or illegally), the target population and its vulnerability, and the method of effective delivery and dissemination. Although these anticoagulants may be absorbed through the skin and lungs, the main route of exposure is ingesting food, water, or adulterated drugs containing the product (Jones et al., 1984; Katona and Wason, 1989; Swigar et al., 1990; Wallace et al., 1990; Exner et al., 1992; Rauch et al., 1994; Gallo, 1998; Corke, 1997; Ross et al., 2019; Tole et al., 2019; Navon et al., 2019; Alipour et al., 2019; Arepally and Ortel, 2019; Boyack and Opsha, 2019; Kelkar et al., 2018; Riley et al., 2019; Panigrahi et al., 2018; Hussain et al., 2018; Moritz et al., 2018). Superwarfarin poisoning may result in a large number of casualties if these substances are ingested (Palmer et al., 1999; Baker et al., 2002; EPA, 2005; POISINDEX, 2007; HSDB, 2008).

Superwarfarin rodenticides are used to kill urban and agricultural pests. They are now less available to the general public (EPA, 2019) but are readily available to pest controllers and are easy to obtain and conceal, so they may pose a risk of being used as chemical warfare agents. These rodenticides are available as meal bait packs, pellets, minipellets, blocks, miniblocks, wax blocks, liquid bait formulations, tracking powder, concentrate, and perhaps other formulations (POISINDEX, 2007).

17.2 Background

Anticoagulants were discovered in the early 20th century after livestock had eaten moldy sweet clover contaminated with bis-hydroxycoumarin and died of hemorrhagic disease. Newer long-acting warfarin derivatives such as brodifacoum, bromadiolone, diphacinone, and chlorophacinone can produce profound and prolonged anticoagulation and bleeding after a latency period that generally lasts 24−72 h (FDA, 1985; Smolinske et al., 1989; Routh et al., 1991; Exner et al., 1992) (Table 17.1).

In the 1940s, a small British pharmaceutical company suggested that dicoumarol might have rodenticidal properties. Trials carried out by Armour and Barnett (1950) confirmed the idea and started the era of anticoagulant rodenticides. Warfarin was the first anticoagulant rodenticide; it was introduced into the market shortly after World War II and became widely used in many countries. Other anticoagulant compounds with potency similar to that of warfarin were also synthesized. These early anticoagulant rodenticides have often been called *first-generation anticoagulant rodenticides.* These compounds generally have moderate toxicity, with acute LD_{50} values ranging from 10 to 50 mg/kg body weight (Table 17.2).

The first-generation compounds often needed continuous bait exposure for rodent control. Many rodent species developed a resistance to warfarin (Jackson et al., 1975),

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TABLE 17.1 Some commercial products containing superwarfarins.

Name	Molecular formula	Commercial names
Brodifacoum CAS: 56073-10-0	C_{31}-H_{23}-Br-O_3	D-Con Mouse-Prufe I & II, Havoc, Klerat, Ratak Plus, Talon G, Void Finale, Folgorat, Matikus, Mouser, Rodend, Volak, Volid
Difenacoum CAS: 56073-07-5	C_{31}-H_{24}-O_3	Compo, Diphenacoum, Frunax DS, Matrak, Neosorexa, Rastop, Ratak, Ratrick, Silo
Bromadiolone CAS: 28772-56-7	C_{30}-H_{23}-Br-O_4	Apobas, Bromard, Bromone, Bromatrol, Bromorat, Contrac, Deadline, Hurex, Lanirat, Maki, Morfaron, Musal, Maki, Ramortal, Ratimon, Rodine-c, Slaymore, Super-caid, Toidon
Diphacinone CAS: 82-66-6	C_{23}-H_{16}-O_3	Diphacine, Ditrac, Gold Crest, Kill-Ko, P.C.Q., Promar, Ramik, Rat Killer, Rodent Cake, and Tomcat
Chlorophacinone CAS: 3691-35-8	C_{23}-H_{15}-Cl-O_3	Caid, Liphadione, Microsul, Ramucide, Ratomet, Raviac, Rozol, Topidox
Difethialone[a] CAS: 104653-34-1	C_{31}-H_{23}-Br-O_2-S	None to report
Pindone[a] CAS: 83-26-1	C_{14}-H_{14}-O_3	Pestanal, Pindone, Pival, Pivalyn, Pivalyl Valone, Tri-ban
Coumatetralyl[a] CAS: 5836-29-3	C_{19}-H_{16}-O_3	Racumin
Coumafuryl[a] CAS: 117-52-2	C_{17}-H_{14}-O_5	Fumarin, Tomarin
Valone CAS: 83-28-3	C_{14}-H_{14}-O_3	None to report
Flocoumafen[a] CAS: 90035-08-8	C_{33}-H_{25}-F_3-O_4	None to report

Available forms include: meal bait packs, pellets, minipellets, blocks, miniblocks, wax blocks, liquid bait formulations, and tracking powder.
[a]*No longer produced or used in the United States.*

TABLE 17.2 The oral LD_{50} values (mg/kg body weight) of some anticoagulant rodenticides.

Animals	Bromadiolone	Brodifacoum	Difenacoum
Rat (acute)	0.65	0.27	1.8
Rat (chronic)	(0.06–0.14) × 5	(0.05–0.08)	0.15 × 5
Mouse	0.99	0.4	0.8
Rabbit	1.0	0.2	2.0
Pig	3.0	10.0	80.0
Dog	10.0	3.5	50.0
Cat	25.0	25.0	100.0
Chicken	5.0	10.0–20.0	50.0
Guinea pig	2.8	–	–
Opossum	–	0.17	–
Sheep	–	10.0	100.0

presumably due to continued exposure and widespread use. Consequently, new chemical structures were synthesized and used as anticoagulant rodenticides. These newer compounds are generally more toxic than warfarin, with acute LD_{50}s of 0.2–3.9 mg/kg body weight. For example, a bait concentration of only 50 ppm of brodifacoum is adequate to control the population in a single feeding for most rodents and noncommensal species (Matolesy et al., 1988).

These newer compounds were called *second-generation anticoagulant rodenticides* and are often now referred to as *superwarfarins* in the contemporary medical literature (Routh et al., 1991; Exner et al., 1992; Rauch et al., 1994; Hui et al., 1996; Sharma and Bentley, 2005; Dolin et al., 2006).

During the past 30 years, there have been hundreds of articles published in medical literature relating to the clinical assessment, laboratory testing, and treatment of human and nonhuman patients exposed to superwarfarins. A great number of these articles are about children under 6 years old who accidentally ingested small amounts of these products and, in most cases, did not experience adverse effects (Brands et al., 1995; Ingels et al., 2002; AAP, 2003; Osterhoudt and Henretig, 2003). A few cases describe coagulopathies, and some fatalities, in patients who intentionally ingested large amounts of the substance (Wallace et al., 1990; Casner, 1998; Walker and Beach, 2002; AAPCC, 2006). Most of the health hazard is associated with ingestion of superwarfarins (Bruno et al., 2000; POISINDEX, 2007). Many publications concern nonhuman animals (Newton et al., 1990; Stone et al., 1999). Virtually all animal exposures are due to accidental direct or indirect ingestion. The great majority of domestic animal exposures are in dogs. Many of these cases require referral to a veterinarian for clinical evaluation and often treatment (DuVall et al., 1989; Hornfeldt and Phearman, 1996; McConnico et al., 1997; Robben et al., 1997; Borst and Counotte, 2002). Occasionally gastric decontamination will be indicated; coagulopathies frequently respond to vitamin K_1 therapy.

Warfarin and dicoumarol found application as both oral anticoagulants and as rodenticides. Sweet clover requires the action of molds to form dicoumarol; giant fennel does not. Giant fennel (*Ferula communis*) grows in Mediterranean countries. It has a naturally occurring anticoagulant effect. An association between the plant and anticoagulation was first reported in the 1950s (Costa, 1950a,b; Carta, 1951). It was further investigated in Italy (Corticelli and Deiana, 1957; Corticelli et al., 1957; Mazzetti and Cappelletti, 1957; Cannava, 1958), and then in Israel (Shlosberg and Egyed, 1983). The anticoagulant activity of the plant in Morocco has also been reviewed (Lamnaouer, 1999).

Warfarin and its congeners are still used as therapeutic agents. Oral anticoagulants available therapeutically in Europe include warfarin, phenprocoumaron, and nicoumalone—also called acenocoumarol (Shetty et al., 1993). Oral anticoagulants are used therapeutically to reduce thromboembolic events. Warfarin examples include a reduction in catheter-related thrombosis (Magagnoli et al., 2006); early venous thrombosis after operations (Pan et al., 2005), including hip surgery; atrial fibrillation (Reiffel, 2000); and myocardial infarction (Asperger and Jursic, 1970). A number of adverse events have been recognized, most of which are related to drug interactions (Dayton and Perel, 1971).

An association between vitamin K_1 and coagulopathies was made in the mid-1930s (Dam, 1935; Fieser et al., 1939). Soon thereafter, Karl Paul Link reported the discovery of dicoumarol in moldy hay (Last, 2002). Naturally occurring coumarin in sweet-clover hay is converted by fungi to dicoumarol. Dicoumarol was found to be the causative agent of the disease, so the elements needed for the disease were coumarin-containing plant material plus mold growth. Subsequently, a range of molecules were synthesized, and one of these, warfarin, became the most popular (Duxbury and Poller, 2001). Warfarin takes its name in part from the Wisconsin Alumni Research Foundation and the rest from coumarin.

17.2.1 AAPCC data on superwarfarins

Every year, tens of thousands of accidental ingestions of long-acting anticoagulant rodenticides (LAARs) are reported worldwide in medical literature. These include the annual report from the American Association of Poison Control Centers (AAPCC). Table 17.3 summarizes exposures, reasons, and deaths associated with LAARs reported by the AAPCC in recent years. All fatalities were in adults who committed intentional suicide.

17.3 Classification of superwarfarins

Anticoagulant rodenticides are also categorized by chemical structure. The chemical structure of the currently marketed products fits in one of two chemical classes: 4-hydroxycoumarins and indanediones.

17.3.1 4-Hydroxycoumarins

This group of compounds has a 4-hydroxycoumarin ring with different side-chain substituents at the 3-position. Commonly used superwarfarin anticoagulant rodenticides in this group are bromadiolone, brodifacoum, coumatetralyl, coumafuryl, and difenacoum. Brodifacoum, difenacoum, and bromadiolone are three of the most commonly used rodenticides around the world. These rodenticides share most of their physical and chemical characteristics, as well as their mechanism of toxicity, and the medical management is the same for all superwarfarins. Generally brodifacoum has longer elimination kinetics.

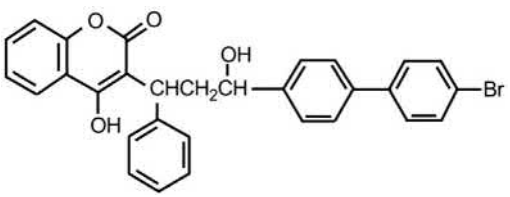
TABLE 17.3 Number of exposures to long-acting anticoagulant rodenticides and deaths reported by the American Association of Poison Control Centers—National Poison Center Annual Reports 2012–2017.

Year	No. of exposures to long-acting anticoagulant rodenticides	Reason: unintentional	Reason: intentional	Reason: other	Deaths
2012[a]	9299	8887	310	70	1
2013[a]	8510	8101	292	77	1
2014[b]	8372	7960	301	76	3
2015[c]	7125	6796	222	72	1
2016[d]	5453	5198	164	53	0
2017[e]	4810	4612	106	55	1

[a]*Mowry, J.B., Spyker, D.A., Cantilena Jr., L.R., Bailey, J.E., Ford, M., 2013. 2012 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 30th Annual Report. Clin. Toxicol. (Phila.) 51 (10), 949–1229. PubMed PMID: 24359283.*
[b]*Mowry, J.B., Spyker, D.A., Brooks, D.E., McMillan, N., Schauben, J.L., 2015. 2014 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 32nd Annual Report. Clin. Toxicol. (Phila.) 53 (10), 962–1147. PubMed PMID: 26624241.*
[c]*Mowry, J.B., Spyker, D.A., Brooks, D.E., Zimmerman, A., Schauben, J.L., 2016. 2015 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 33rd Annual Report. Clin. Toxicol. (Phila.) 54 (10), 924–1109. PubMed PMID: 28004588.*
[d]*Gummin, D.D., Mowry, J.B., Spyker, D.A., Brooks, D.E., Fraser, M.O., Banner, W., et al., 2017. 2016 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 34th Annual Report. Clin. Toxicol. (Phila.) 55 (10), 1072–1252. PubMed PMID: 29185815.*
[e]*Gummin, D.D., Mowry, J.B., Spyker, D.A., Brooks, D.E., Osterthaler, K.M., et al., 2018. 2017 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 35th Annual Report. Clin. Toxicol. (Phila.) 21, 1–203. PubMed PMID: 30576252.*

17.3.1.1 Bromadiolone

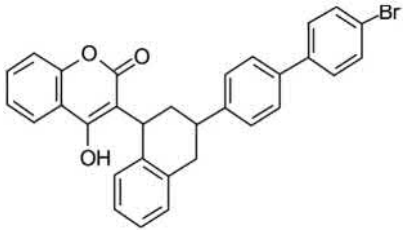
Chemical formula: $C_{30}H_{23}BrO_4$



Bromadiolone [3-(3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenyl propyl)-4-hydroxycoumarin] was synthesized and marketed by the French company Lipha SA during the mid-1970s. It is used widely for control of commensal and field rodents in many countries. Technical-grade bromadiolone is 97% pure. It is a yellowish powder and stable up to 200°C (Chalermchaikit et al., 1993). It is very soluble in dimethylformamide (730 g/L), but less soluble in ethyl acetate (25 g/L) and ethanol (8.2 g/L), and sparingly soluble in water (0.019 g/L). Bromadiolone is considered more palatable to rodents than most other anticoagulants. Its concentration in baits is usually 50 ppm (Chalermchaikit et al., 1993). Although bromadiolone is considered a second-generation anticoagulant rodenticide, resistance has been reported in *Rattus norvegicus* and *Mus musculus* in the United Kingdom and Denmark (Rowe et al., 1981; Lund, 1984; IPCS, 1995d).

17.3.1.2 Brodifacoum

Chemical formula: $C_{31}H_{23}BrO_3$



Brodifacoum [3-(3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro naphth-1-yl)-4-hydroxycoumarin] is one of the more potent second-generation anticoagulant rodenticides. It was first introduced in 1977 by Sorex Ltd. of London, and then developed by the Imperial Chemicals Incorporated (ICI) Plant Protection Division (Chalermchaikit et al., 1993).

Pure brodifacoum is an off-white to fawn-colored powder with a solubility of 6–20 g/L in acetone, 3 g/L in chloroform, 0.6–6 g/L in benzene, and less than 10 mg/L water. It is very stable in the environment with no loss after 30 days of exposure to direct sunlight (Chalermchaikit et al., 1993).

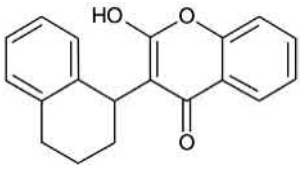
Brodifacoum has been marketed in several countries for the control of a wide range of rodent pest species. It is available as a 0.005% pellet for rat and mouse control, a smaller 0.001% pellet for field rodent control, and as 29 g wax blocks for sewer rat control. It is the only anticoagulant rodenticide found to produce 100% mortality in most

rodent species after only a 24 h dose (Chalermchaikit et al., 1993). Brodifacoum was effective against warfarin-resistant rats and mice in 1984, but resistance has been reported (Lund, 1984).

Species variation in susceptibility exists. Dogs are susceptible and are commonly exposed to potentially toxic quantities of brodifacoum (Chalermchaikit et al., 1993).

17.3.1.3 Coumatetralyl

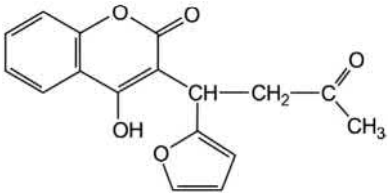
Chemical formula: $C_{19}H_{16}O_3$



Coumatetralyl [3-(alpha-tetralyl)-4-hydroxycoumarin] was introduced by Bayer AG with the trademark name of Racumin. It has been used for commensal rodent control in many countries. It is formulated as a dry bait (0.0375%), a liquid bait of its sodium salt, and a 0.75% tracking dust (Chalermchaikit et al., 1993). Pure coumatetralyl is a colorless powder that is stable at temperatures below 150°C. Its solubility is 20–50 g/L in propan-2-ol, 50–100 g/L in methylene dichloride, and 4 mg/L in water. The acute and chronic LD_{50}s of *R. norvegicus* are 16.5 and 0.3 mg/kg for five consecutive doses, respectively. Chickens are somewhat resistant to coumatetralyl, with a chronic LD_{50} of 50 mg/kg for eight consecutive doses. Signs did not appear in fish until the concentration of coumatetralyl reached 1000 mg/L in water (Chalermchaikit et al., 1993). Despite its low toxicity, it is reported to be more effective than warfarin against *R. norvegicus*, apparently due to a higher palatability. Coumatetralyl was introduced after the detection of warfarin-resistant rat populations and showed considerable success for a number of years, but resistance has been reported in the United Kingdom and Denmark (Rowe and Redfern, 1968; Lund, 1984).

17.3.1.4 Coumafuryl

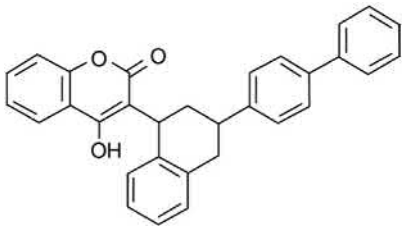
Chemical formula: $C_{17}H_{14}O_5$



Coumafuryl [3-(alpha-acetonylfurfuryl)-4-hydroxycoumarin] is a German anticoagulant, introduced in 1952, used at 0.025%–0.05% in baits. Its toxicity is considered equal to warfarin for *R. norvegicus*, but slightly less efficient against *M. musculus*. The chronic LD_{50} in *R. norvegicus* is 1.4 mg/kg for five repeated doses. Cats and dogs seem to be almost as susceptible as rats, with dogs being killed by 2 mg/kg for five repeated doses and cats by 10 mg/kg for four repeated doses (Chalermchaikit et al., 1993).

17.3.1.5 Difenacoum

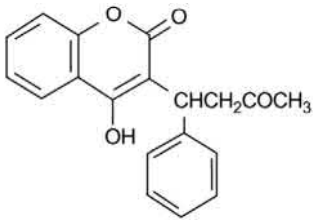
Chemical formula: $C_{31}H_{24}O_3$



Difenacoum [3-(3-*p*-diphenyl-1,2,3,4-hydronaphth-1-yl)-4-hydroxycoumarin] was synthesized in the United Kingdom and marketed in 1975 by Sorex Ltd. under the trademark Neosorexa, and by ICI Plant Protection Division under the trademark Ratak as a 0.005% pelleted bait, and as a wax block. Pure difenacoum is an off-white powder with a solubility of greater than 50 g/L in acetone, 600 mg/L in benzene, and less than 10 mg/L in water. It is more toxic than warfarin but less palatable (IPCS, 1995c). Difenacoum is still effective against many populations of warfarin-resistant rats (Desideri et al., 1979), but resistance has been reported in the United Kingdom (Greaves et al., 1982).

17.3.1.6 Warfarin

Chemical formula: $C_{19}H_{16}O_4$



Warfarin [3-(a-acetonylbenzyl)-4-hydroxycoumarin] was the first anticoagulant rodenticide. It was introduced shortly after World War II by the Wisconsin Alumni Research Foundation. Warfarin is still used widely, especially for the control of *R. norvegicus* in areas where resistance has not yet developed. In its racemic form, warfarin is colorless and crystalline, insoluble in water but readily soluble in acetone,

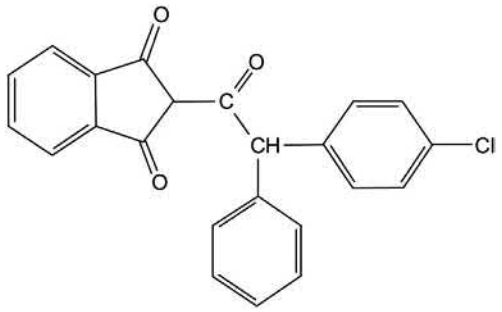
dioxane, and moderately soluble in alcohols. Warfarin is formulated as dry bait (0.005%−0.05%), liquid bait, based on the sodium salt, and a tracking dust (0.5%−1.0%). It is generally applied as the S-isomer, which is 10 times more potent than the R-isomer. The acute and chronic LD_{50}s for *R. norvegicus* are around 10−12 and 0.75 mg/kg for five repeated doses, respectively (Colvin and Wang, 1974). Warfarin is sometimes combined with an antibacterial agent, sulfaquinoxaline, to reduce the bacterial production of vitamin K in the rat intestine, but the effectiveness of this combination is not well established. Warfarin is considered one of the safest anticoagulants, as far as nontarget animals are concerned. Serious resistance problems have been reported in Europe. It has recently been evaluated against sewer rats in London (Channon et al., 2000).

17.3.2 Indanediones

This group of compounds has a 1,3-indanedione structure with different side-chain substituents at the 2-position. The most common superwarfarins in this group are chlorophacinone and diphacinone.

17.3.2.1 Chlorophacinone

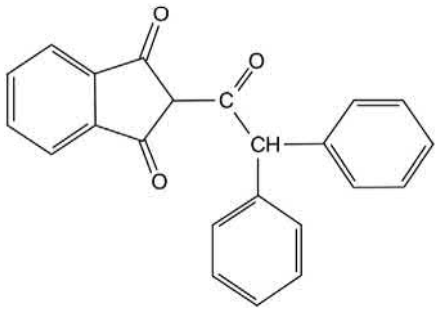
Molecular formula: $C_{23}H_{15}ClO_3$



Chlorophacinone [2-(alpha-4-chlorophenyl-a-phenylacetyl)-1,3-indandione] was introduced in the mid-1960s by Lipha SA at concentrations of 0.05% in baits and 0.2% in tracking dust. Pure chlorophacinone is a yellow crystalline solid that is very soluble in acetone, ethanol, and ethyl acetate, but is only somewhat soluble in water. It is quite stable and resistant to weathering. Chlorophacinone does not induce bait-shyness and is compatible with cereals, fruits, roots, and other potential bait substances. Its acute LD_{50} in *R. norvegicus* is about 20.5 mg/kg, which is less toxic than warfarin, but it has a stronger initial effect on rats and mice. For control of house mice populations, a prolonged feeding period is needed. Chlorophacinone may not be effective against warfarin-resistant rodents (Chalermchaikit et al., 1993).

17.3.2.2 Diphacinone

Molecular formula: $C_{23}H_{16}O_3$



Diphacinone (2-diphenylacetyl-1,3-indandione) is an old anticoagulant rodenticide introduced by Vesicol Chemical Corp. and the Upjohn Co. It has been produced and used primarily in the United States as a 0.005% dry or liquid bait. Pure diphacinone is a yellow powder that is very soluble in chloroform (204 g/kg), toluene (73 g/kg), xylene (50 g/kg), and acetone (29 g/kg), but only somewhat soluble in water (0.30 g/L). It will decompose in water due to sunlight. The acute LD_{50}s in *R. norvegicus* are 22.7 mg/kg in females and 43.3 mg/kg in males. It is more toxic than warfarin to rats, mice, and dogs, but its palatability is somewhat lower. Diphacinone may not be effective against some warfarin-resistant rodents (Chalermchaikit et al., 1993). The anticoagulant rodenticides are marketed to have efficacy against a number of target pest species.

17.4 Toxicokinetics

17.4.1 Absorption, metabolism, and excretion in laboratory animals and humans

Superwarfarins are generally well absorbed from the gastrointestinal tract. Almost 90% is absorbed, with peak plasma concentrations often occurring within 12 h of ingestion. Binding to plasma proteins may prolong distribution and half-life. Toxicity after dermal or respiratory exposure is reported, but rare (Boermans et al., 1991; Berry et al., 2000; Spiller et al., 2003).

The metabolism and elimination of the *trans*-isomer is more rapid than that of the *cis*-isomer. Elimination from the liver is biphasic, with an initial rapid phase of 3 days and a slower phase with a half-life of 120−130 days. The liver is the major organ for detection of the unchanged parent compound. The major route of elimination in different species after oral administration is via the feces. The urine is a very minor route of elimination (Watt et al., 2005).

17.5 Mechanism of action

The mechanism of action of all anticoagulant rodenticides is similar to that of warfarin, specifically inhibition of vitamin K_1 epoxide reductase (Park et al., 1979; Leck and Park, 1981; Breckenridge et al., 1985). In the coagulation cascade, clotting factors II, VII, IX, and X must bind calcium ions to be active in clot formation. The Ca^{2+}-binding ability requires converting glutamyl residues on these clotting factors to carboxyl glutamyl residues by the process of carboxylation. This carboxylation uses vitamin K_1 hydroquinone as a cofactor. This vitamin K-dependent carboxylase reaction converts vitamin K_1 hydroquinone to its epoxide form, vitamin K_1 2,3-epoxide. Normally vitamin K_1 2,3-epoxide is reduced to the original vitamin K_1 (phylloquinone) by epoxide reductase, thus being "recycled." The anticoagulant rodenticides interfere with vitamin K_1 epoxide reductase, resulting in the depletion of vitamin K_1 and subsequently impairing the synthesis of normal clotting factors II, VII, IX, and X (Craciun et al., 1997, 1998). Clinical coagulopathy soon follows the depletion of active clotting factors in circulation. In the dog, these clotting factors have plasma half-lives of 41, 6.2, 13.9, and 16.5 h, respectively. The coagulation system may function reasonably well until about 3–5 days after ingestion (depending on the compound and dose), when the natural decay of clotting factors occurs. The interrelationship of vitamin K, prothrombin, and gamma-carboxyglutamic acid is reviewed in Stenflo (1978). The interaction of warfarin and vitamin K is reviewed in Suttie (1990).

17.6 Toxicity

17.6.1 Clinical effects: signs and symptoms

Clinical signs and symptoms of acute intoxication by superwarfarins range from a mild tendency to bleed (in less severe poisoning cases) to severe coagulopathy. Mild bleeding tendencies often show themselves clinically as nose or gum bleeding, hemoptysis, ecchymosis, bloody or melenotic stools, hematuria, abdominal or flank pain, enhanced bruising, and ventral hematomas (in animals). Severe bleeding leads to shock and death. Internal and external bleeding are the most frequent clinical signs, followed by tachycardia and hypotension, then failure of multiple organs due to substantial blood loss. The onset of clinical signs is normally not evident until a few days after absorption (Baker et al., 2002; Tsutaoka et al., 2003).

17.6.1.1 Animal toxicology

Clinical signs are usually delayed until 24–36 h after ingestion. The most common signs include, dyspnea, weakness, lethargy, anorexia, hematuria, or melena. Other signs, such as pale mucous membranes, bleeding from the nose and gums, and generalized bruising, also may be noticeable. Internal bleeding also causes generalized pain; lameness from bleeding into a joint; bleeding into the thorax or abdomen, brain, or pericardium; or sudden death (Braithwaite, 1982; Berny et al., 1995; Munday and Thompson, 2003).

17.6.1.2 Pediatric exposures

The great majority of human exposures are children under the age of 6 due to accidental/unintentional ingestion. Children usually do not require any medical intervention or routine follow-up laboratory studies and can be adequately managed by poison control centers with home observation and parent education (Mullins et al., 2000; Ingels et al., 2002; Kanabar and Volans, 2002; Shepherd et al., 2002). Children with accidental ingestions of a pellet may often be managed without gastric decontamination or prophylactic vitamin K. Laboratory testing for coagulopathy should be reserved for cases involving clinically evident bleeding abnormalities (Ingels et al., 2002).

A small number of reported cases of children have presented with mild to moderate hematological effects, requiring minimal intervention (Smolinske et al., 1989; Watts et al., 1990; Babcock et al., 1993; Travis et al., 1993; Golej et al., 2001; Osterhoudt and Henretig, 2003).

On the other hand, intentional suicidal ingestion of large amounts of product conveys a greater risk of severe toxicity and increased mortality, normally requiring referral to a healthcare facility for examination and treatment (Ingels et al., 2002).

17.6.1.3 Adult exposures

The great majority of adult exposures to rodenticides are due to deliberate acute or chronic ingestion. Bleeding disorders may persist for 6 weeks to many months. Serious poisoning has been reported in adults with massive overdoses. These long-acting anticoagulants have produced rapid and persistent bleeding due to hypoprothrombinemia (Chong et al., 1986; Hoffman et al., 1988; Katona and Wason, 1989; Wallace et al., 1990; Routh et al., 1991; Barnett et al., 1992; Exner et al., 1992; Mack, 1994; Morgan et al., 1996; McCarthy et al., 1997; Gallo, 1998; Berry et al., 2000). A risk of spontaneous abortion and transplacental distribution has been reported. (Lipton and Klass, 1984; Zurawski and Kelly, 1997; Nelson et al., 2006; Mehlhaff et al., 2013; Yan et al., 2013; Rutović et al., 2013).

The severity of the intoxication depends on the amount of rodenticide ingested, preexisting comorbidity, and coingestion of other toxic substances (Seidelmann et al., 1995;

Palmer et al., 1999; Stanziale et al., 1997; Tecimer and Yam, 1997; Walker and Beach, 2002). Fatalities are usually due to intentional suicidal ingestion of large amounts. Bleeding disorders and organ failure have been described in adults due to deliberate acute and chronic surreptitious ingestion. See Table 17.3 for a summary of exposures, reasons, and deaths reported by the AAPCC in recent years

17.6.1.4 Household pets and farm animal exposures

Household pets and farm animals may be accidentally exposed to rodenticides. The possible effects on nontarget organisms can be considered in two types: direct poisoning and secondary poisoning. Secondary poisoning is generally considered to occur after an animal has consumed anticoagulant rodenticide-poisoned target species, such as rodents. The most common type of exposure is direct poisoning by eating a cereal-based bait containing the rodenticide. The most commonly affected household pets are dogs, followed by cats, hamsters, rabbits, and pet birds (Redfern and Gill, 1980; Park and Leck, 1982; Boermans et al., 1991; Woody et al., 1992; Hornfeldt and Phearman, 1996; Peterson and Streeter 1996; McConnico et al., 1997; Robben et al., 1997, 1998; Munday and Thompson, 2003; Radi and Thompson, 2004).

Cats may be more resistant to the toxic effects of brodifacoum and difenacoum than dogs. Cases of abortion and hemorrhage in sheep and goats after misuse of brodifacoum have been reported (Jones, 1996; Watt et al., 2005). Recent case reports suspect anticoagulant rodenticide toxicosis in pregnant women (Mehlhaff et al., 2013; Yan et al., 2013) and a whelping dog (Fitzgerald et al., 2018).

17.6.1.5 Nontarget wildlife exposures

Nontarget wildlife also may be exposed to rodenticides. The possible effects on nontarget organisms can also be considered in two types: direct poisoning and secondary poisoning. The potential for secondary poisoning is more likely in carnivorous wildlife (Mathur and Prakash, 1980; Mahmoud and Redfern, 1981; Greaves et al., 1982; DuVall et al., 1989; Newton et al., 1990; James et al., 1998; Borst and Counotte, 2002; Eason et al., 2002). The most commonly affected species are birds such as great horned owls, barn owls, eastern screech owls, golden eagles, red-tailed hawks, Cooper's hawks, and crows. Bird species varied in their susceptibility. Other wild animals also may be exposed, such as polecats, wildcats, and foxes. Brodifacoum was highly toxic for fish when tested as a technical material.

17.6.1.6 Laboratory/monitoring and general recommendations

Superwarfarins lower the active blood concentrations of the vitamin K-dependent clotting factors II, VII, IX, and X; this results in prolongation of prothrombin time (PT) and partial thromboplastin time (PTT). PT and PTT should be repeated at least twice daily until normal PT and PTT levels are established. Also, the blood clotting time and the bleeding time should be measured. Blood is often demonstrable in the excreta. Secondary hypochromic or microcytic anemia may be marked (Goldfrank et al., 2002; Nelson et al., 2006). A PT 24−48 h after exposure in asymptomatic children with accidental ingestions of large or unknown amounts should be obtained. In adults with deliberate ingestions and children with clinical evidence of bleeding, an initial PT and PTT should be obtained and then repeated at 24 and 48 h after ingestion (Manoguerra and Cobaugh, 2005).

Evaluation of a point-of-care anticoagulant rodenticide test for dogs has been reported (Istvan et al., 2014).

17.6.1.7 Analytical methods

A number of analytical methods have been reported for detecting anticoagulant rodenticides in various matrices. Early fluorimetric methods were used to detect warfarin in serum (Vesell and Shivley, 1974; Fasco et al., 1977; Hanna et al., 1978; Lee et al., 1981) and GLC for warfarin (Mildha et al., 1974).

Warfarin-specific methods were generally inadequate for subsequent anticoagulant rodenticides, so a number of other methods were developed. These methods include thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), mass spectroscopy (MS), and antibody-mediated tests. Coumarin anticoagulant rodenticides were initially detected using TLC (Lau-Cam and Chu-Fong, 1972; Mallet et al., 1973). A high-performance TLC method with an estimated detection limit of 200 ppb and 87% recovery from liver was reported (Berny et al., 1995).

Early HPLC methods focused on an individual chemical. For example, methods to detect chlorophacinone in formulations (Vigh et al., 1981), brodifacoum in serum (Murphy et al., 1989), brodifacoum (Ray et al., 1989), bromadiolone (Subbiah et al., 2005), chlorophacionone (Hunter, 1985), difethiolone (Goldade et al., 1998), and difenacoum (Mundy and Machin, 1977) in tissue have been reported.

Subsequently, methods were developed to look for all anticoagulant rodenticides in the same sample at the same time. An initial method succeeded in extracting and detecting eight anticoagulant rodenticides in serum and liver using fluorescence and ultraviolet (UV) detection.

Samples were extracted with acetonitrile then cleaned up on solid-phase columns. Four hydroxycoumarins were detected by fluorescence with excitation at 318 nm and emission at 390 nm. The indandiones were detected at 285 nm. An extraction recovery of 75% from serum and 69% from liver was reported. Hydroxycoumarins may be detected down to about 1 ng/mL of serum and 1 ng/g of liver, and indandiones down to 10 ng/mL of serum and 10 ng/g of liver (Felice and Murphy, 1989; Felice et al., 1991; Chalermchaikit et al., 1993). Another HPLC method for detecting brodifacoum in serum and liver using difenacoum as the internal standard has been reported (O'Bryan and Constable, 1991). There is also a method for the simultaneous detection of five superwarfarin rodenticides in human serum (Kuijpers et al., 1995). Other serum methods have been reported; for example, detection limits of 3–12 ng/mL for fluorescence and 20–75 ng/mL for UV detection (Mura et al., 1992; Kuijpers et al., 1995; McCarthy et al., 1997; Feng et al., 1999).

Tissue methods include a solid-phase cartridge extraction from liver, with recoveries ranging from 52% for difenacoum to 78% for warfarin. The limit of detection is 10 ppb for warfarin and difenacoum and 110 ppb for chlorophacinone (Addison, 1982; Jones, 1996; Fauconnet et al., 1997).

HPLC methods have also been published to distinguish *cis*- and *trans*-isomers of difenacoum with detection limits of 5 ng/mL (Kelly et al., 1993). An early, interesting approach was to use a postcolumn pH shift to enhance fluorescence detection (Hunter, 1985). Several earlier HPLC methods have also been reported (AOAC, 1976a,b; Hunter, 1983): diphacinone (Bullard et al., 1975, 1976), fluorescence for bromadiolone (Deepa and Mishra, 2005), brodifacoum (Fu et al., 2006), brodifacoum in tissues (Hoogenboom and Rammell, 1983), difenacoum (Hadler and Shadbolt, 1975), determination of Rozol in parafinized formulations (Kawano and Chang, 1980), and bromadiolone in tissues (Nahas, 1986).

A recent method uses diode-array detection (Yang et al., 2001). Contemporary confirmatory methods use MS, for example, liquid chromatography-electrospray ionization-mass spectroscopy (LC-EIS-MS) has been reported for the analysis of 10 anticoagulant rodenticides with a quantity limit of about 5 μg/L (Grobosch et al., 2006). Other recent methods use LC-MS-MS for unknown drugs, including warfarin (Marquet et al., 2003), and LC-ESI-MS and HPLC UV to detect anticoagulant rodenticides as low as 20 ng on a column (Mesmer and Flurer, 2000). Recently reported methods tend to use liquid chromatography with one or more MS units (Robinson and Sisco, 2018; Qiao et al., 2018; Smith et al., 2017; López-García et al., 2017; Saito-Shida et al., 2016; Maršálek et al., 2015; Jagerdeo et al., 2015; Bidny et al., 2015).

A prior immunoassay was used to detect diphacinone and chlorophacionone (Mount et al., 1988). Enantiomers of warfarin, coumachlor, and coumafuryl can be separated chromatographically (Armstrong et al., 1993).

Serum concentration of dogs with anticoagulant rodenticide poisoning ranged from less than 10 to 851 ng/L for brodifacoum, difethialone, and difenacoum (Robben et al., 1998).

Animal samples are routinely analyzed in veterinary diagnostic laboratories. For example, the Texas Veterinary Medical Diagnostic Laboratory, in College Station, TX (http://tvmdl.tamu.edu/), performs such analyses. A list of other laboratories performing anticoagulant rodenticide analyses on animal samples can be obtained from the American Association of Veterinary Laboratory Diagnosticians (www.aavld.org). Human samples are generally analyzed at either the National Medical Services Laboratory in Willow Grove, PA, or the Medtox Scientific Laboratories in St Paul, MN.

17.7 General treatment recommendations

17.7.1 Referral to healthcare facilities

In the case of a suspected terrorist act, misuse, intentional criminal, or any deliberate intentional suicidal ingestion, or when the amount ingested is either large or indeterminate, the patient should be referred to a healthcare facility for clinical and laboratory assessment, as well as treatment if necessary (Manoguerra and Cobaugh, 2005; POISINDEX, 2007). Therapeutic approaches to anticoagulant rodenticide toxicoses have been reviewed (Schulman and Furie, 2015).

A recent review of dogs with coagulopathies reports that 60.9% tested positive for anticoagulant rodenticides, 31.3% had neoplasia, 14.6% immune-mediated disease, and 10.4% gastrointestinal bleeding. Dogs with anticoagulant rodenticide intoxication had the best prognosis, with a survival rate of 98.7% (Waddell et al., 2013).

17.7.2 Home observation criteria

Accidental ingestion of a small amount (less than a few pellets) can normally be adequately managed at home by either poison control centers or a healthcare professional with home observation and parent education. Usually, these types of exposures do not require any medical intervention or routine follow-up laboratory studies. Gastric decontamination has no effect on the clinical outcome after "taste" (one-fifth of a spoonful) amounts are ingested by children (Mullins et al., 2000; Kanabar and Volans, 2002; Shepherd et al., 2002).

If the amount ingested by a child is a "moderate amount" (defined as more than a handful, or a mouthful),

or is questionably high, then the parent should contact a physician or call a local poison control center for instructions on how to induce emesis (Tenenbein et al., 1987; AAP, 2003).

The amount ingested by nonhuman animals is rarely known. When the amount ingested is unknown, or cannot be estimated, then a local poison control center or veterinarian should be contacted for instructions on how to induce emesis and to obtain a 24- to 48-h blood test (PT) to determine the need for treatment with vitamin K_1 (Murphy et al., 1989; Munday and Thompson, 2003).

17.7.3 Treatment at healthcare facilities

A PT should be obtained 48 h after exposure in asymptomatic children with accidental ingestion of a suspected large amount of rodenticide (Babcock et al., 1993; Berry et al., 2000). Adults with intentional ingestion and children with clinical evidence of bleeding should obtain an initial PT and PTT, repeated at 24 and 48 h after ingestion (Goldfrank et al., 2002). If any significant prolongation or evidence of bleeding is observed, PT should be repeated every 6–12 h. Determination of factors II, VII, IX, and X may be abnormal in patients with a normal PT and PTT, which may provide evidence of earlier significant ingestion (Pavlu et al., 2005; Spahr et al., 2007). Hemoglobin and hematocrit should be monitored in patients with clinical evidence of bleeding or significant coagulopathy. Determination of the ABO blood type may be necessary in cases of toxic ingestions and bleeding. Patients may require red blood cell transfusions or the administration of fresh frozen plasma (Bruno et al., 2000; Laposata et al., 2007; Olmos and Lopez, 2007).

17.7.3.1 Emesis

Currently, there is controversy regarding the use of syrup of ipecac. The American Academy of Pediatrics reversed its policy position about its use in poisoning emergencies in children (AAP, 2003). Simultaneously, the AAPCC indicates that syrup of ipecac has a place in therapy, and "concluded that individual practitioners and poison control centers are best able to determine the particular patient population, geographic and other variables that might influence the decision to recommend having ipecac on hand" (Manoguerra and Cobaugh, 2005). For dosing of ipecac, see Table 17.4. The first action for a caregiver of a child who may have ingested one of these rodenticides is to consult with their local poison control center (AAP, 2003) (Table 17.5).

Emesis is contraindicated in patients with a prolonged PT or a bleeding disorder due to the risk of bleeding following ipecac-induced increased intracranial pressure (POISINDEX, 2007). Taste amounts (a few pellets or a bite of one block of bait) do not require emesis. If the amount is more than two mouthfuls or one block of bait, or if an unknown amount is ingested, then emesis is most effective if initiated within 30 min to 1 h from the time of ingestion. The decision to induce emesis or not is often controversial; so must be carefully considered. It could be most appropriate in prehospital settings and is not recommended once the patient is in the emergency room (Chyka and Seger, 1997; Krenzelok et al., 1997).

Contraindications: Patients with a bleeding disorder, particularly those under treatment with anticoagulants or with histories of chronic, long-acting anticoagulant ingestion, are at risk from gastrointestinal and central nervous system (CNS) bleeding from ipecac-induced emesis. The administration of activated charcoal is preferred when large amounts have been ingested or chronic ingestion has occurred. Also, it is contraindicated if there is a risk for choking or aspiration, CNS excitation or depression, coma, seizures, or signs of oral, pharyngeal, or esophageal irritation (Golej et al., 2001; Goldfrank et al., 2002).

Before or after ipecac is administered, patients should be encouraged to drink water. Adults are given approximately 8 oz (240 mL), and children 4–8 oz (120–240 mL) (Goldfrank et al., 2002; POISINDEX, 2007).

TABLE 17.4 Dosing of ipecac.

Adult[a]	15–30 mL
Adolescent[a]	15–30 mL
Child 1–12 years old	15 mL
Child 6–12 months	Dose: 5–10 mL (position child in left lateral decubitus position to reduce risk of aspiration)
Child under 6 months of age	*Not* recommended for prehospital use

[a]*Katona and Wason (1989).*

TABLE 17.5 Dosing of activated charcoal (Chyka and Seger, 1997).

Charcoal dose recommended to dilute 240 mL of water per 30 g charcoal (FDA, 1985)	
Adults and adolescents	50–100 g
Children aged 1–12 years	25–50 g
Infants up to 1 year old	1 g/kg of body weight

17.7.3.2 Activated charcoal

For patients with a potentially toxic ingestion who are awake and able to protect their airway, activated charcoal diluted in water may be administered before going to the hospital. It is more effective when administered within 1 h after ingestion. It is recommended to dilute the mixture with 240 mL of water per 30 g charcoal (Chyka and Seger, 1997).

In patients who are at risk of onset of seizures or depression, activated charcoal should be administered by medical or paramedical personnel capable of airway management to prevent aspiration in the event of spontaneous emesis (POISINDEX, 2007).

Use of a cathartic is not routinely recommended, as there is no evidence that cathartics reduce drug absorption, and cathartics can cause adverse effects such as nausea, vomiting, abdominal cramps, electrolyte imbalances, and occasionally hypotension (Chyka and Seger, 1997; Golej et al., 2001). Use of cholestyramine has been reported to increase survival in a rabbit brodifacoum model (Lindeblad et al., 2018).

17.7.3.3 Gastric lavage

Gastric lavage is recommended within 1−2 h after ingestion; but not afterwards, as it may induce bleeding in patients with significant coagulopathy (Brands et al., 1995).

17.7.3.4 Laboratory monitoring

PT and PTT values should be obtained 24 and 48 h postingestion in asymptomatic children with accidental ingestion of a "large amount." Adults with intentional ingestions and children with clinical confirmation of bleeding should obtain initial PT and PTT readings, repeated at 24 and 48 h postingestion, followed by blood type verification (Barnett et al., 1992; Ellenhorn et al., 1997; Robben et al., 1998). If significant prolongation or evidence of bleeding is observed, PT should be repeated every 6−12 h. Determination of factors II, VII, IX, and X may be abnormal in patients with a normal PT and PTT, which may provide support of earlier ingestion. Serial hemoglobin and hematocrit values in patients should be followed with clinical evidence of bleeding or significant coagulopathy (Babcock et al., 1993; Robben et al., 1998). Hematocrit should be monitored every 4 h until the patient is stable (Brands et al., 1995).

Hematest should be performed in stools and vomit for occult blood, and PT and PTT monitored routinely. PT and PTT readings obtained within 48 h postingestion may not be predictive of subsequent coagulopathy (Greeff et al., 1987). A 24 and 48 h PT and PTT, therefore, are recommended every 6−12 h to assess the efficacy of therapy. If prolongation is observed, then PT or international normalized ratio measurements should be repeated (Hoffman et al., 1988; Smolinske et al., 1989).

Antidote: Vitamin K_1 (Phytonadione: AquaMephyton, Mephyton) is the specific antidote and should be administered to any patient with a prolonged PT (Braithwaite, 1982; Bruno et al., 2000; Tsutaoka et al., 2003). Blood and fresh or frozen plasma are recommended if the anticoagulation is severe.

Administration of vitamin K_1 orally is recommended if anticoagulation is excessive. In anemic patients, the hematocrit should be monitored about every 4 h until it is stable. Stools and vomit may also be tested using Hematest (Hornfeldt and Phearman, 1996).

Oral vitamin K_1 may be administered in small doses after the patient has been stabilized. Recommended doses are 15−25 mg p.o. in adults, 5−10 mg in children (Greeff et al., 1987), and 2.5−5 mg/kg body weight in animals. A large daily maintenance dose of vitamin K_1 may be required for prolonged therapy in severe overdoses, particularly in patients in whom vitamin K_1 absorption is variable (Lipton and Klass, 1984; Hoffman et al., 1988; Ross et al., 1992; Murphy, 2012).

Intravenous phytonadione may be instituted in severe cases where rapid correction is needed. *Note*: Anaphylaxis is reported to occur during i.v. injection of vitamin K1, particularly if rapidly injected. The adult dose is a minimum of 10 mg diluted in saline or glucose, injected intravenously (i.v.) at a rate not exceeding 5% of the total dose per minute. Doses should be repeated at intervals of 6−8 h. Initial i.v. doses of 25, 100, 150, 160, and 400 mg have been required in patients actively bleeding (Hoffman et al., 1988; Vogel et al., 1988).

Coagulation factors: Recently, prothrombin complex concentrates (Doyle et al., 2018; Wang et al., 2016) and plasma exchange (Deng and Qiu, 2016) have been reported for anticoagulant rodenticide toxicoses. Autologous blood transfusions in dogs with thoracic or abdominal hemorrhage have been used in dogs in emergency situations (Higgs et al., 2015).

17.8 Concluding remarks and future directions

A large number of industrial and household chemicals, including the superwarfarins, have the potential to be used as chemical warfare or terrorist agents. However, the Federal Insecticide, Fungicide, and Rodenticide Act classifies these rodenticides in the "low-toxicity group." Nevertheless, they may be used to harm and terrorize people through the ingestion of contaminated food, water, or adulterated drugs. Superwarfarins are available to consumers as meal bait packs, pellets, minipellets, blocks, miniblocks, wax blocks, liquid bait formulations, and tracking

powder in diluted to concentrated formulations. The ingestion of small amounts may not cause any bleeding problems. Ingestion of greater amounts or repeated exposure provides increased risk of severe bleeding in 36–48 h. The coagulopathy may last several weeks to months despite vitamin K_1 treatment.

What should be done at this time? Be wary if people from a community that attended the same event, meal, party, or restaurant show similar signs of bleeding disorders. Ingestion of superwarfarins may go unnoticed when they are mixed with food, and signs or symptoms are delayed 36–48 h. Consequently, the victims may not associate the ingestion with the coagulopathy.

Identification of superwarfarins is easy nowadays. Analytical methods for the detection of these products in serum and tissues are readily available. Routine laboratory tests for coagulopathy may help support the need for such analytical chemistry testing. It may be useful to create stockpiles of vitamin K_1, particularly in places where large stocks of superwarfarin rodenticides are used.

Although viral infections manifest with different signs and symptoms, it is possible that members from a community who are victims of superwarfarins may begin to panic, thinking that they were victims of a virus such as Ebola, but the signs and symptoms of superwarfarin ingestion differ from viral infection. Superwarfarin intoxication may have no signs or symptoms other than the appearance of bleeding in the stools, urine, mucous membranes, or the thoracic or abdominal cavities. The Ebola virus causes sudden hemorrhagic fever, weakness, muscle pain, headache, and sore throat, followed by vomiting, diarrhea, rash, limited kidney and liver functions, and both internal and external bleeding. Various hemorrhagic disease of animals also occur; an example of current interest is African swine fever. Anticoagulant rodenticide ingestion should be considered in the differential diagnosis in these cases.

Consequently, good differential diagnostic and laboratory work-up algorithms are needed for both humans and animals. Teams of subject matter experts for both humans and animals may be useful in this regard. Community education programs, for example, fact sheets may be developed to inform residents about superwarfarin rodenticides and other household commercial chemicals that could be used as chemical warfare or terror agents. Identifying those at risk, as well as deciding when they should be informed, may be a useful part of this education campaign. Such a program may also improve security in industrial plants and limit access from outsiders to all industrial and storage facilities where flammable or highly toxic chemicals are stored.

Finally, surveillance of food sources, particularly those derived from livestock, should be assessed.

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Chapter 18

PCBs, dioxins, and furans: human exposure and health effects

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18.1 Introduction

Polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated biphenyls (PBBs), polybrominated dibenzo-*p*-dioxins (PBDDs), and polybrominated dibenzofurans (PBDFs) are members of the group of halogenated aromatic hydrocarbons (HAHs). This group of chemicals has been identified by national and international agencies as priority environmental pollutants posing significant effects on aquatic and terrestrial animals, including humans (Loganathan and Kannan, 1994; Jacobson, 1994; Van den Berg et al., 1998; Kodavanti et al., 2008; International Programme on Chemical Safety (IPCS), 1998). PCBs were produced in large quantities (millions of pounds) for a variety of industrial uses during the 1940s to the late 1970s, especially by developed nations. PBBs were produced as flame retardant mainly before the 1970s. Whereas PCDDs and PBDDs (dioxins), PCDFs and PBDFs (furans) were never produced commercially. These compounds are formed in small quantities as byproducts of combustion and various industrial as well as natural processes (Gabos et al., 2001). Due to their persistent, bioaccumulative, and toxic properties, these residues are found in every component of the global ecosystem (Lipnick et al., 2001; Kodavanti et al., 2008). Some PCBs (coplanar PCBs), dioxins, and furans are three structurally and toxicologically related families of compounds that are classified as the most toxic manmade chemical substances to a variety of animal species, including humans (Tucker et al., 1983; Loganathan et al., 1995; ATSDR, 1998). Production of PCBs was banned during the 1970s, however, already-produced PCBs are still causing environmental and health problems (Loganathan et al., 2008; Sajwan et al., 2008; Loganathan, 2016). Fortunately, these compounds were never used as chemical warfare agents, weapons of mass destruction, or agents of threat or terror by contamination of air, water, food/feed, etc. However, inadvertent poisonings of these chemicals have caused significant environmental and health problems (Yusho Support Center, 2007; Loganathan, 2012; Kodavanti and Loganathan, 2012). In this chapter, the historical background, chemical characteristics, analysis, pathways of human exposure to these compounds, and toxic effects associated with exposures are presented.

18.2 Historical background

PCBs were first synthesized in the early 1880s (Schmidt and Schultz, 1881) and commercial production began in 1929. Biphenyls were reacted with Cl_2 in the presence of ferric chloride catalyst, and some of the hydrogen atoms were replaced by chlorine atoms. PCBs were produced as complex mixtures potentially containing 209 congeners formed by chlorinating biphenyl with from 1 to 10 chlorines (Fig. 18.1). The amount of chlorination of biphenyls corresponded to the duration of the chlorination process. For example, Aroclor 1221, 1242, 1248, 1254, 1260, and 1268 are commercial preparations that were formerly produced by the Monsanto Chemical Company in the United States (St. Louis, MO) that contain 21%, 42%, 48%, 54%, 60%, and 68% chlorine by weight, respectively, as indicated by the last two digits in the numerical designation (Giesy and Kannan, 1998). The PCB mixture formulations were different depending on the country of origin, and were produced in Germany (Clofen), France (Phenoclor and Pyralene), Japan (Kanechlor), Italy (Fenclor), Russia (Sovol), and Czechoslovakia (Delor).

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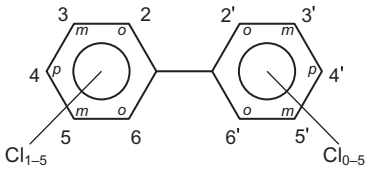
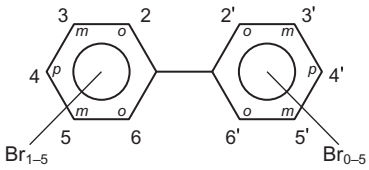
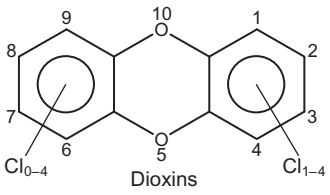
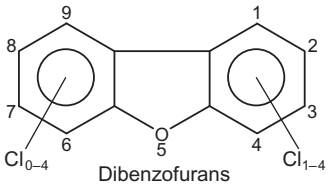
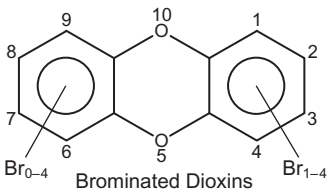
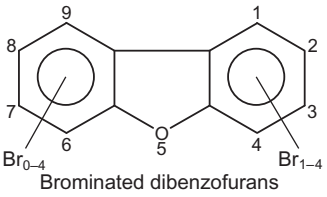
Compound	Molecular Wt.	Chemical structure
PCBs	188–498	
PBBs	232–943	
PCDDs	218–460	
PCDFs	202–444	
PBDDs	263–816	
PBDFs	247–800	

FIGURE 18.1 Generalized structures of PCBs, dioxins, and furans.

PCB mixtures were produced for a variety of uses such as fluids in electrical transformers, capacitors, heat transfer fluids, hydraulic fluids, lubricating and cutting oils, and as additives in plastics, paints, pigments, copying paper, printing inks, adhesives, and sealants (Loganathan et al., 1989; Safe, 1990; Grimm et al., 2017; Dhakal et al., 2018). PBBs were once synthesized widely as flame retardants for plastics. In 1973, several thousand pounds of PBB-based flame retardant, FireMaster BP-6, were accidentally mixed with livestock feed in Michigan, USA, and caused damage to chickens, cattle, and pigs (Carter, 1976). PBBs are now controlled under the RoHS directive in EU and hexabromobiphenyl, one of their homologue, is listed in the Stockholm Convention on Persistent Organic Pollutants. Dioxins and furans are not produced deliberately, but are produced unintentionally as byproducts of the combustion of organic matter in the presence of chlorine. Dioxins and furans consist of 135 possible chlorinated (or brominated) dibenzofuran and 75 chlorinated (or brominated) dibenzo-*p*-dioxins with from one to eight chlorine (bromine) substituents (Fig. 18.2). PCDDs/DFs are found as byproducts during the manufacture of some industrial chemicals such as PCBs, polychlorinated naphthalenes, chlorinated phenols, chlorinated

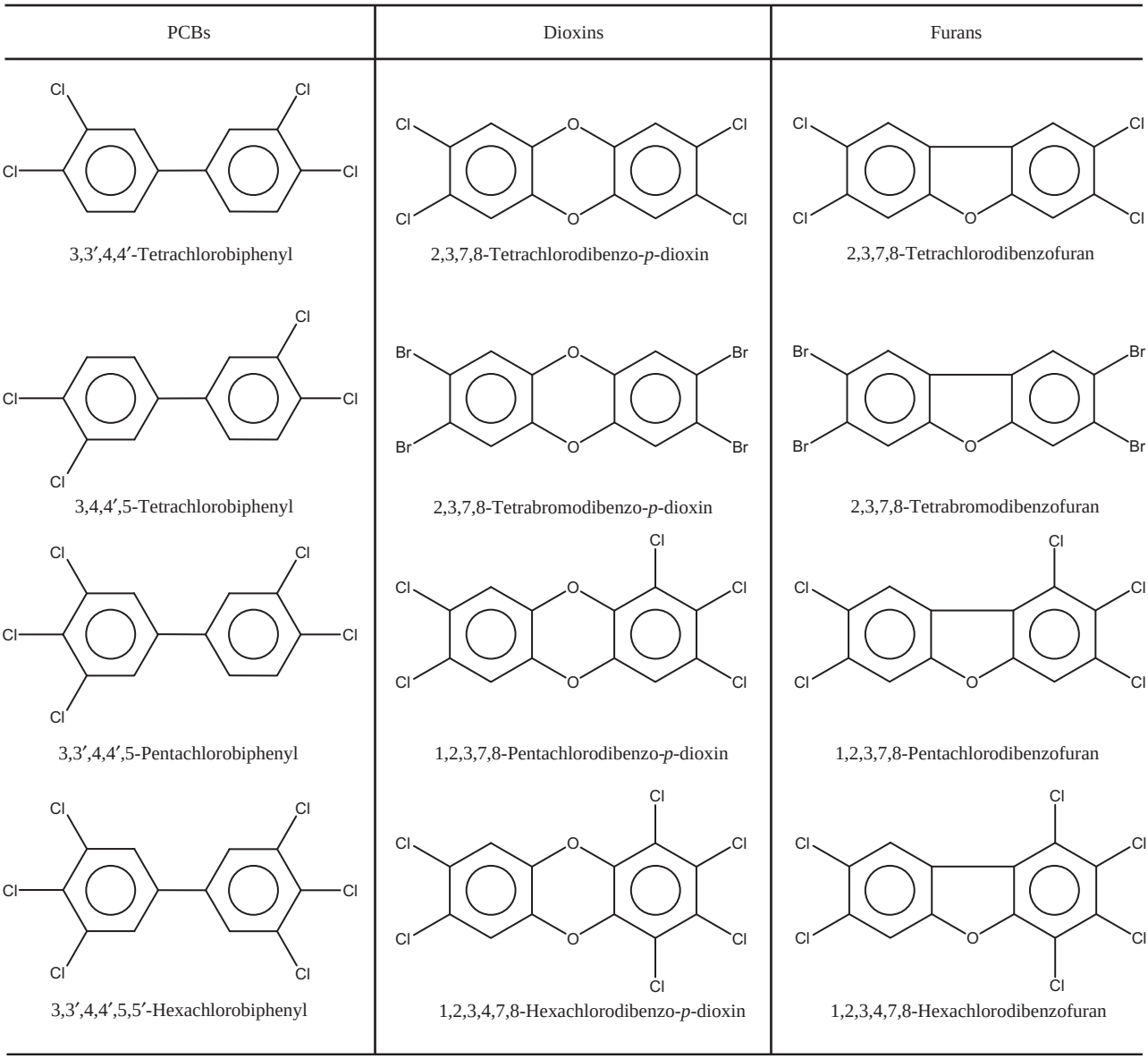


FIGURE 18.2 Structures of highly toxic PCBs, dioxins, and furans.

phenoxyacids, polychlorinated diphenyl ethers, polyvinyl chlorides, and chlorinated phenoxy-2-phenols (Hutzinger et al., 1985; Hryhorczuk et al., 1986; ATSDR, 2001; Masunaga et al., 2001a). Similarly, PBDDs/DFs are found as byproducts in brominated organic chemicals such as brominated flame retardants [polybrominated diphenyl ethers (PBDEs), decabromobiphenyl, 1,2-bis(tribromophenoxy)ethane, tetrabromobisphenol A (TBBPA), etc.] (International Programme on Chemical Safety, 1998). These compounds are also formed during incineration of industrial and municipal waste, forest fires, fireplaces, and combustion engines (Loganathan et al., 1997; Feil and Larsen, 2001; Villa et al., 2016). Because of anthropogenic as well as natural processes, PCBs, dioxins, and furans are widely dispersed in the global environment and their presence has been reported in air, water, soil, sediment, and aquatic and terrestrial organisms including human tissues (Loganathan and Kannan, 1994; Giesy and Kannan, 1998; Masunaga et al., 2001b; Ogura et al., 2001).

18.3 Human exposure to PCBs, PCDDs, and PCDFs

Direct human exposure to PCBs/PCDFs has occurred due to inadvertent poisoning due to consumption of PCB-contaminated food such as Yusho and Yucheng poisoning

(oil disease) in Japan and Taiwan in 1968 and 1979, respectively. Yusho Support Center (2007) states that "39 years have passed since the outbreak of YUSHO, the PCB/dioxin tragedy – the most unprecedented incident in the history of mankind whereby people ingested toxic chemicals unknowingly, directly through food." The outbreak of a strange disease "Yusho" (Kanemi Oil Poisoning) occurred in the western part of Japan in 1968. The major symptoms and signs of the disease consisted of acne-like eruptions, pigmentation of the skin, nails, and conjunctivas, increased discharge from eyes, and numbness in the limbs (Yao et al., 2002; Yusho Support Center, 2007). The epidemic was identified later (1969) to be an unprecedented mass food poisoning caused by the ingestion of commercial brand rice oil that had been contaminated by PCBs and their related compounds. The number of people who reported having ingested the rice oil was about 14,000, and 1867 persons were designated as Yusho victims. A similar outbreak of "oil disease" occurred in Taiwan in 1979. Toxicological studies revealed that PCDF congeners, including 2,3,4,7,8-pentachlorodibenzofuran (which were byproducts of PCBs and also formed by heat during the use of PCBs as a heat exchange oil), played an important role in the pathogenesis of the disease. Research conducted on Yusho victims revealed harmful effects of the exposure continued for two generations (Yusho Support Center, 2007). A recent study revealed the concentration of 2,3,4,7,8-PeCDF, the most toxic compound in Yusho patients, was significantly correlated with the presence of comedones or acneiform eruption, oral pigmentation, numbness in the hands and legs, and arthralgia (Mitoma et al., 2015).

Direct human exposure to dioxin occurred in southern Vietnam. It was estimated that southern Vietnam has been contaminated by 160–600 kg of dioxin as a result of 80 million liters of defoliant herbicides (Agent Orange, a 50:50 mixture of 2,4,5-T and 2,4-D) being sprayed by the US military over a large area of forests and crops of southern Vietnam from 1962 to 1971 (Westing, 1984; Schecter et al., 2006; Le Hong Thorn et al., 2007). The defoliant was contaminated with a very toxic form of dioxin (TCDD) known to have caused adverse effects on human health. Dioxin may cause harmful effects on the whole body and can affect separately the functioning of systems such as nervous system, immune responses, carcinogenicity, hepatotoxicity, and metabolic and enzyme toxicity (Le Hong Thorn et al., 2007). Recently, Pham and his research group compared the toxic effects of dioxins in mother–newborn pairs from the Bien Hoa airbase, the most contaminated area with dioxin (due to Agent Orange) and Ha Dong district of Ha Noi city, an unexposed area in Vietnam. After studying 288 mother–newborn pairs recruited in 2012 and 2015, who were highly exposed to dioxin, and 120 pairs recruited in 2014 from the unexposed area, the authors found that decreased expressive and composite language scores in boys and motor scores in girls were found in children exposed to TCDD $>=5.5$ pg/g lipid in maternal breast milk (Pham et al., 2019). However, the National Academies of Sciences, Engineering, and Medicine asserted in their 2016 update on Vietnam veterans that the epidemiologic evidence is sufficient to conclude that there are positive associations between exposure to herbicide and health outcomes, such as soft-tissue sarcoma (including heart), non-Hodgkin lymphoma, chronic lymphocytic leukemia (including hairy cell leukemia and other chromic B-cell leukemias), Hodgkin lymphoma, and chloracne (National Academies of Sciences, Engineering, and Medicine, 2016).

FIGURE 18.3 Dioxin poisoning. Ukrainian former Prime Minister and presidential candidate Viktor Yushchenko, with his face disfigured by illness due to dioxin poisoning. *Photo: http://news.bbc.co.uk/2/hi/europe/4105035.stm (accessed 18.07.19.).*

Another example of direct human poisoning of dioxin was food poisoning of Mr. Viktor Yushchenko (Fig. 18.3), Ukrainian presidential candidate, in 2004. The dioxin poisoning caused a mysterious illness that resulted in his face becoming pockmarked and ashen (BBC News, December 17, 2004: http://news.bbc.co.uk/2/hi/europe/4105035.stm). Manahan (1992) classified dioxin (2,3,7,8-TCDD) as super toxic in comparison with other know toxic substances (Table 18.1).

18.4 Physicochemical properties and global distribution

The unusual industrial versatility of PCBs was directly related to physical and chemical properties which include

TABLE 18.1 Toxicity level of various chemical compounds.

Toxicity level	Compounds	LD_{50} estimated from laboratory animals[a] (mg/kg)
Slightly toxic	Ethyl alcohol	10,000
	Sodium chloride	5000
Moderately toxic	Malathion (organophosphorus pesticide)	1000
	Chlordane (termite exterminator)	500
Very toxic	Heptachlor (pesticide)	100
Extremely toxic	Parathion (pesticide)	10
	Dioxin (2,3,7,8-TCDD)	5 (Hamster)
	Tetraethyl pyrophosphate (pesticide, raticide)	1
Super toxic	Tetrodotoxin (toxin of blowfish)	0.1
	Dioxin (2,3,7,8-TCDD)	0.0006 (Guinea pig)
	Botulin (toxin of botulinum)	0.00001

[a]*LD_{50} are rough values estimated from oral-dose experiments on laboratory animals (usually rats). Unit: mg/kg body weight.*
Source: Prepared from Manahan, S.E., 1992. Toxicological Chemistry. CRC Press, p. 464.

resistance to acids and bases, compatibility with organic materials, resistance to oxidation and reduction, excellent insulating properties, nonflammability, and thermal stability (Hutzinger et al., 1985). The physical and chemical stability of PCBs are vital to industrial applications and the same properties have been responsible for global environment contamination. In addition, multimedia releases and volatility lead to long-range environmental transport, both via water and the atmosphere, resulting in widespread environmental contamination of humans and wildlife at sites distant from their use (Lipnick and Muir, 2001; Loganathan et al., 2008). In PCBs, dioxins, and furans, the properties vary widely and depend on the number and position of chlorine (or bromine) atoms attached to the molecule. In general, vapor pressure, water solubility, and biodegradability decrease with an increasing number of chlorine atoms. Lipophilicity adsorption capacity shows a reverse trend (Loganathan and Kannan, 1994). Because of these unique properties, PCBs, dioxins, and furans have been detected in air and water (rivers, lake ecosystems) (Pearson et al., 1997; Loganathan et al., 1998a,b, 2001). Apart from this, these compounds were recorded in fish, birds, and marine mammals of several other ecosystems, such as the Atlantic, Baltic, and Pacific oceans and Swedish environments (Kawano et al., 1988; Loganathan et al., 1999). They have been identified in processed fish and other food products (Kannan et al., 1997; Patandin et al., 1999). Further, the residues of these contaminants were found in human adipose tissue, blood, and milk, and also in numerous other matrices (Loganathan et al., 1993, 1998a,b, 1999; Petreas et al., 2001; Czaja et al., 2001).

The comparison of PCDD/DFs and PBDD/DFs in terms of toxic equivalents (TEQs) calculated based on WHO-TEF (World Health Organization's toxic equivalent factors) in pooled human milk samples from 17 countries showed that PBDD/DF levels are significantly lower than the PCDD/DF (mean PBDD/DF:PCDD/DF-TEQ ratio was 0.13 and its range was 0.06 − 0.25), indicating that exposure to PCDDs/DFs is much greater than PBDD/DFs and more important to human health (Kotz et al., 2005). This was supported by a study on human adipose tissue and plasma, in which TEQs for PBDD/DFs (0.2−0.8 pg TEQ/g lipid) were 1% − 15% those of PCDD/DFs-TEQs (Jogsten et al., 2010) and the contribution of PCDD/DFs, dioxin-like PCBs, and PBDDs/DFs to total TEQ in human blood were 47%, 31%, and 21%, respectively, on a median basis (Fromme et al., 2016). In some environmental media, however, the contribution of PBDD/DFs to TEQs cannot be neglected. The median TEQ contributions of PBDFs, PCDDs, PCDFs, non-*ortho* Co-PCBs, PCNs, and mono-ortho Co-PCBs determined by GC-HRMS in indoor dusts to the TEQ evaluated by in vitro bioassay (CALUX-TEQs) (range 38−1400, median 160 pg TEQ/g) were 17%, 14%, 8.8%, 0.98%, 0.10%, and 0.019%, respectively (Suzuki et al., 2010). Analyses of 12 PBDD/DF congeners by GC-HRMS in composited, archived biosolids collected from 94 wastewater treatment plants in the United States revealed that the TEQ of PBDD/DFs was 162 (range: 15 − 672) ng TEQ/kg and at 75% (range: 12% − 96%) of the total TEQ (Venkatesan and Halden, 2014).

Because of the large production and indiscriminate use of PCBs in industry, these contaminants extended

their boundaries of distribution over the global environment and this was evidenced by their detection even in pristine environmental media and biota such as the Arctic and Antarctic atmosphere, hydrosphere, and biosphere (Muir et al., 1988; Corsolini et al., 2002; Kumar et al., 2002). The discovery of widespread environmental occurrence, the increased general environmental concern, and the apparent link to carcinogenesis and other health disorders prompted a public outcry which resulted in the prohibition of PCBs as well as chlorinated pesticides in several developed nations during the early 1970s. The following section deals with the chemical analysis of PCBs, dioxins, and furans in environmental and biological samples.

18.5 Analytical methods

PCBs and PCDDs/DFs consist of a total of 419 individual congeners and PBDDs/DFs have 210 congeners. In addition, dioxins and furans with mixed chlorine and bromine substitution can occur. These congeners have quite a variety of toxicity and some of these congeners, especially planar dioxins, furans, and dioxin-like PCBs (non-*ortho* chlorine substituted coplanar PCBs) are extremely toxic, even at very low concentrations (Table 18.1). Therefore, determination of some toxic congeners to a very low concentration (parts per trillion) has become important.

Congener-specific determination was required for those congeners that have toxic equivalency factors (TEFs), namely 2,3,7,8-chlrine substituted dioxins and dioxin-like PCBs (Table 18.2). Thus, these compounds are analyzed using a high-resolution gas chromatograph high-resolution mass spectrometer (HRGC-HRMS). A schematic flow chart of the representative analysis procedure for environmental and biological samples is shown in Fig. 18.3 based on standard analytical methods, such as US EPA methods 1613, 1668, 1668a, and JIS K0312 (US EPA, 1994, 1997, 1999; JISC, 2008).

Biological samples are either freeze-dried or dewatered (homogenized) with anhydrous sodium sulfate salt and spiked with internal standards (cleanup spike) and then extracted using a Soxhlet apparatus. Obtained extracts are concentrated and their solvents are changed to an appropriate solvent such as hexane. They then go through a series of cleanup procedures to remove lipid and other interfering chemicals. Then eluates are concentrated again and spiked with internal standards. The prepared samples are injected into HRGC-HRMS and monitored by multiple ion monitoring mode.

TABLE 18.2 Toxic equivalency factors (TEFs) for dioxins and dioxin-like PCBs.

Congener	TEF				
	I-TEF	WHO-TEF of 1998 (Van den Berg et al., 1998)			WHO-TEQ of 2006 (Van den Berg et al., 2006)
	Human	Human and mammals	Fish	Bird	Human and mammals
PCDDs					
2,3,7,8-TCDD	1	1	1	1	1
1,2,3,7,8-PeCDD	0.5	1	1	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1	0.5	0.05	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1	0.01	0.01	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1	0.01	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.1	0.01	0.001	<0.001	0.01
OctaCDD	0.001	0.0001	–	–	0.0003
PCDFs					
2,3,7,8-TCDF	0.1	0.1	0.05	1	0.1
1,2,3,7,8-PeCDF	0.05	0.05	0.05	0.1	0.03
2,3,4,7,8-PeCDF	0.5	0.5	0.5	1	0.3
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1

(*Continued*)

TABLE 18.2 (Continued)

Congener	TEF				
	I-TEF	WHO-TEF of 1998 (Van den Berg et al., 1998)			WHO-TEQ of 2006 (Van den Berg et al., 2006)
	Human	Human and mammals	Fish	Bird	Human and mammals
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01	0.01	0.01
OctaCDF	0.001	0.0001	0.0001	0.0001	0.0003
Non-ortho-PCBs					
3,4,4′,5-TeCB (#81)		0.0001	0.0005	0.1	0.0003
3,3′,4,4′-TeCB (#77)		0.0001	0.0001	0.05	0.0001
3,3′,4,4′,5-PeCB (#126)		0.1	0.005	0.1	0.1
3,3′,4,4′,5,5′-HxCB (#169)		0.01	0.00005	0.001	0.03
Mono-ortho-PCBs					
2,3,3′,4,4′-PeCB (#105)		0.0001	<0.000005	0.0001	0.00003
2,3,4,4′,5-PeCB (#114)		0.0005	<0.000005	0.0001	0.00003
2,3′,4,4′,5-PeCB (#118)		0.0001	<0.000005	0.00001	0.00003
2′,3,4,4′,5-PeCB (#123)		0.0001	<0.000005	0.00001	0.00003
2,3,3′,4,4′,5-HxCB (#156)		0.0005	<0.000005	0.0001	0.00003
2,3,3′,4,4′,5′-HxCB (#157)		0.0005	<0.000005	0.0001	0.00003
2,3′,4,4′,5,5′-HxCB (#167)		0.00001	<0.000005	0.00001	0.00003
2,3,3′,4,4′,5,5′-HpCB (#189)		0.0001	<0.000005	0.00001	0.00003

Concentrations are calculated by the isotope dilution method. Stable isotope-labeled target compounds are used as internal standards and spiked into samples. Calibration with internal standards and determination by isotope dilution are necessary to obtain reliable data under very low concentrations and after repeated pretreatment and cleanup procedures.

Solid samples, such as soil and sediment, are air-dried and extracted by a Soxhlet/Dean-Stark apparatus. Aqueous samples are separated into solids and filtrates by filters. Solids are Soxhlet extracted similarly to biological samples and filtrates are liquid–liquid extracted. Then, these extracts are cleaned up and injected into HRGC-HRMS, similarly to biological samples.

In cases when congener-specific information is not necessary, other simpler methods can be used. EPA methods 608 and 8082a use GC/EDC to determine the concentration of PCBs in terms of Aroclor (Federal Register,

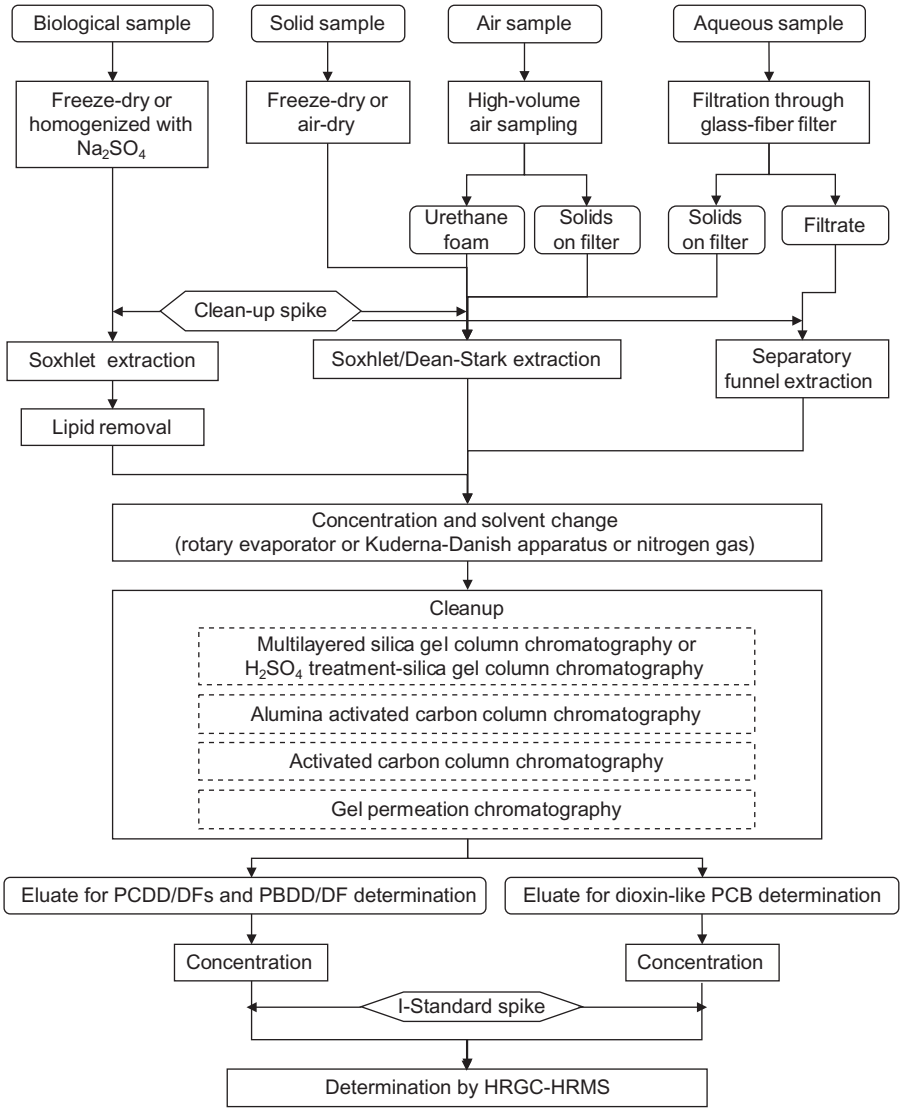


FIGURE 18.4 Analytical procedure of dioxins, furans, and dioxin-like PCBs.

1984; US EPA, 2007). EPA method 680 (US EPA, 1985) uses GC/MS (low-resolution mass spectrometer) to determine the homolog concentration of PCBs (Fig. 18.4).

18.6 Mechanism of action and toxicity

The toxicities of PCBs, PCDDs/DFs, and PBDDs/DFs are complicated by the presence of a large number of congeners, each with its own toxicity. Commercial PCB mixtures elicit a broad spectrum of toxic responses that are dependent on several factors, including chlorine content, purity, dose, species, age and sex, and duration of exposure. Immunotoxicity, carcinogenicity, and developmental toxicity as well as biochemical effects of commercial PCB mixtures have been studied extensively in various laboratory animals, fish, and wildlife (Giesy and Kannan, 1998). Several studies have confirmed the common receptor-mediated mechanism of action of toxic halogenated aromatics and has resulted in the development of a structure−activity relationship for this class of chemicals (Safe, 1990). The most toxic halogenated aromatic is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is assigned the maximum toxicity factor of 1, the relative toxicities of individual halogenated aromatics have been determined relative to TCDD (i.e., toxic equivalents). The 17 congeners of PCDD/DFs and the 12 congeners of dioxin-like PCBs are assigned TEFs lower than TCDD (Table 18.2). In June 1997, the World Health Organization established the levels of toxicity factors (WHO-TEFs) to be applied to evaluating the risks for humans and animals (Van den Berg et al., 2006). The

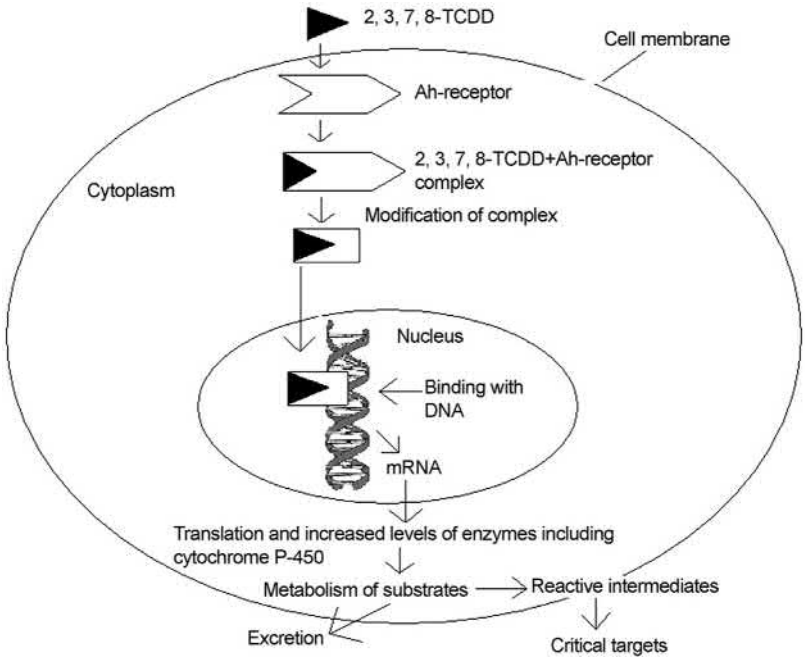


FIGURE 18.5 Possible mechanism of toxic action of dioxins and dioxin-like PCBs.

WHO consultation set the tolerable daily intake (TDI) between 1 and 4 pg TEQ/kg body weight, emphasizing that the aim was to lower the TDI level to under 1 pg TEQ/kg body weight (Guerzoni and Raccanelli, 2004). The most toxic PCB congeners are those that have chlorine substitution in most of the non-*ortho* positions, such as 3, 4, and 5 in each ring. These coplanar PCB congeners (Fig. 18.2) are structurally similar to highly toxic 2,3,7,8-TCDD and exhibit similar toxic responses (Ah-receptor-mediated toxicity) (Fig. 18.5). There are no WHO-TEFs for 2,3,7,8-substituted PBDDs/DFs and dioxin-like PBBs, yet. However, use of the same TEF values for structurally corresponding brominated and chlorinated congeners for human risk assessment was recommended by a joint WHO and United Nations Environment Programme (UNEP) expert consultation in 2011 (Van den Berg at al., 2013). 2,3,7,8-TCDD and structurally related halogenated aromatic compounds induce a variety of microsomal enzymes primarily in the liver. 2,3,7,8-TCDD evokes dose-related induction of cytochrome-P-450-associated AHH (aryl hydrocarbon hydroxylase) activity. The most widely studied of these responses are induction of AHH and EROD (markers of CYP1A activity) in mammalian cell cultures and in laboratory rodents (Goldstein and Safe, 1989).

Ah-receptor-mediated toxicity resulted in a wide range of biological responses, including alterations in metabolic pathways, body weight loss, thymic atrophy, impaired immune responses, hepatotoxicity, chloracne and related skin lesions, developmental and reproductive effects, and neoplasia.

18.7 Concluding remarks and future directions

PCBs, dioxins, and furans are persistent organic pollutants (POPs) which have negative effects on the environment and health of humans including skin toxicity, immunotoxicity, neurotoxicity, negative effects on reproduction, teratogenicity, endocrine disruption, and a predisposition to cancer. A major pathway of exposure to these chemicals is through consumption of food contaminated by PCBs dioxins and furans. The Committee of Experts on Food of the European Commission proposed a dose called the "tolerable weekly intake" given by a total of dioxins and PCBs of 14 pg TEQ/kg of body weight, which is an average of 2 pg TEQ/day/kg of body weight (Guerzoni and Raccanelli, 2004). By reducing the environmental contamination, we can diminish the food chain accumulation and ultimately we can reduce the intake levels of PCBs and dioxins and their toxic health effects.

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Chapter 19

Polycyclic aromatic hydrocarbons: implications for developmental, molecular, and behavioral neurotoxicity

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19.1 Introduction

Airborne polycyclic aromatic hydrocarbons (PAHs) arise from incomplete combustion of carbon-based fuels. They are common in the environment, arising from multiple sources including industrial emissions from coal-fired power plants, motor vehicle emissions (especially diesel exhaust), tobacco smoke, and dietary sources such as charbroiled foods (Banks et al., 2019). Exposure to PAHs is rapidly being recognized as a global problem, particularly in major urban centers in the United States and developing nations such as China, where urban environmental protections tend to be weaker and where biomass is used as a source of indoor fuel (Zhang and Tao, 2009). In these situations, these contaminants accumulate to toxic levels in the body within a short period of time and enter the environment rapidly (ATSDR, 1995; WHO, 2010). Chronic exposure to even low concentrations of PAHs causes long-lasting damage, such as cancer, infertility, and neurotoxicity to humans and wildlife (Banks et al., 2019).

The goal of this chapter is to encourage research activity into both novel intervention strategies and therapeutic approaches to mitigate the neurotoxicity associated with in utero exposure to PAHs as combustion-derived agents of urban industrialization and warfare (acts of arson, which includes setting of oil well fires, terrorism, burning of ammunition dumps, etc.; Lederman et al., 2004; Fent et al., 2014; Masiol et al., 2016; Joyeta, 2018). The focus of this chapter is to highlight current epidemiological data pertaining to the known neurobehavioral outcomes from PAH exposures, as well as supporting data in animals. An attempt is made to suggest potential operative pathways contributing to PAH exposure-induced neurodevelopmental deficits.

A critical review of the post-September 11, 2001, literature has resulted in a specific focus on studies that have revealed critical neural signaling/activity pathways and the identification of primary targets of PAH toxicity, as well as toxicity modifiers. It is hoped that this chapter will stimulate multidisciplinary efforts to look at the conduct of temporal and spatial integrative analyses of developmental processes affected by exposure to PAHs.

19.2 Background

19.2.1 Epidemiological evidence for the negative effects of PAHs on pregnant women

A number of cohort studies have been undertaken to assess pregnancy outcomes and early life impacts

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associated with the September 11, 2001, attack and other airborne PAH exposures. To study the acute exposure of pregnant women either working in the World Trade Center (WTC) or residing in the communities of lower Manhattan on September 11, 2001, a Mount Sinai Hospital cohort was assembled (Berkowitz et al., 2003). Of the 187 recruited pregnant women, 12 (about 6%) were inside the WTC towers at the time of attack, and an additional 121 (65%) were within eight blocks (Wolff et al., 2005). A comparison group ($n = 2367$) consisted of all private patients not known to have been near the WTC who delivered at Mount Sinai Hospital during the same time period.

Another cohort of 300 women who lived, worked, or delivered their infants in lower Manhattan in the weeks and months after September 11 were recruited to examine chronic exposure after the attacks (Lederman et al., 2004). These women were followed by the Columbia Center for Children's Environmental Health. The New York City Department of Health and Mental Hygiene and the Agency for Toxic Substances and Disease Registry (ATSDR) established the World Trade Center Health Registry, a voluntary registry to prospectively monitor physical and mental health in the aftermath of the attacks among those with a high probability of direct exposure, including pregnant women (Lipkind et al., 2010).

In recognition of the impact of environmental toxicants encountered in utero and the disparities in adverse birth outcomes among inner-city, minority populations, the Columbia Center for Children's Environmental Health established a prospective cohort to follow pregnant women and their children longitudinally (Perera et al., 2003). Nonsmoking Dominican and African-American women residing in three NYC neighborhoods were monitored for exposure to environmental tobacco smoke (ETS), airborne PAHs, and pesticides. A parallel prospective cohort was assembled in Krakow, Poland, with the same eligibility criteria, study design, and air-monitoring methods (Jedrychowski et al., 2009). In Tongliang, China, the effects from PAH exposure secondary to coal combustion were studied prior to and after the shutdown of a coal-fired power plant (Perera et al., 2005a; Tang et al., 2006).

19.2.2 Conclusion from prospective epidemiology cohort studies

Table 19.1 outlines the main findings from follow-up studies of the cohorts described earlier in this chapter. Analysis of the 187 exposed women in the Mount Sinai WTC cohort revealed nearly a twofold increase in the odds of intrauterine growth retardation in their babies [IUGR; adjusted odds ratio (AOR) 1.90; 95% CI, 1.05−3.46] (Berkowitz et al., 2003). Biologically plausible causes of IUGR in these babies include exposures to fine particulate matter (PM) and PAHs. Previous studies have found associations between particulate air pollution and IUGR (Dejmek et al., 1999; Bobak et al., 2001). Other investigations have linked air pollution to preterm births (Ritz et al., 2000). Additionally, high levels of PAH-DNA adducts in umbilical cord leukocytes are associated with reduced birth size (Perera et al., 1998).

Among the cohort of 300 nonsmoking women who were pregnant on September 11, 2001, and lived within a 2-mile radius of the WTC during the following month, a number of decrements were found in their babies compared to the babies of other pregnant women studied. Infants born to exposed women showed a decrease of 122 g in birth weight and 0.74 cm in birth length after adjusting for gestational duration, sociodemographic, and biomedical risk factors (Lederman et al., 2004). Infants born to women who were in their first trimester at the time of the attacks had significantly shorter gestation (−3.6 days) and smaller head circumference (−0.48 cm) compared to infants born to mothers exposed during their second or third trimester. The 446 births recorded in the World Trade Center Registry also showed a variation in mean gestational age by trimester on September 11, in addition to differences in birth weight (Lipkind et al., 2010). Analysis of births from previous years, however, revealed that the effect of trimester observed may have been due to a seasonal effect unrelated to exposure. It is important to note that the odds of low birth weight and preterm delivery were higher in registry-linked births to mothers that probably had posttraumatic stress disorder (PTSD; Lipkind et al., 2010).

19.2.3 Effects of maternal stress

In addition to PAH exposure during pregnancy, maternal stress associated with a terrorist attack or disaster has been shown to affect birth outcomes and later neurocognitive development. Among the 187 women in the Mount Sinai cohort who were pregnant and living or working within close proximity to the WTC on September 11, 52 women completed at least one psychological assessment prior to the delivery of their child. In this analysis, Engel et al. (2005) found that posttraumatic stress symptomatology (PTSS) was associated with decrements in infant head circumference at birth. These decrements could later influence neurocognitive development, although follow-up on this cohort has not yet been reported.

Analysis of live singleton births to women enrolled in the World Trade Center Health Registry between September 11, 2001, and October 31, 2002, also found an apparent effect of maternal stress. When comparing women enrolled in the registry (who were exposed) to

TABLE 19.1 Summary of findings from studies examining cognitive and developmental outcomes resulting from in utero exposure to PAHs.

Cohort and authors	Age	Development measure	Findings
Tongliang, coal-fired power plant pre- and post-shutdown (Perera et al., 2008)	2 years of age	GDS	Significant association between elevated cord blood adducts and decreased motor development quotient and average development quotient in 2002 cohort (pre-shutdown), but not 2005 (post-shutdown)
Tongliang, coal-fired power plant pre- and post-shutdown (Tang et al., 2013)	Summary of birth–2 years	GDS	Children born after the power plant shutdown had greater head circumference ($P = .001$), reduced levels of cord blood adducts ($P < .001$), and were exposed to lower levels of ambient PAHs compared to the cohort born prior to shutdown ($P = .01$)
Tongliang, coal-fired power plant pre- and post-shutdown (Tang et al., 2014)	Summary of birth–2 years	GDS	An inverse association was found between BDNF and PAH-DNA adducts in cord blood. Developmental quotient scores were positively associated with BDNF levels
A subset of the CCCEH cohort pregnant on 9/11 or became pregnant in following month ($n = 300$) (Perera et al., 2007)	3 years	BSID-II	A significant interaction between cord blood DNA adducts and in utero exposure to ETS on MDI score ($P = .02$), but cord blood adducts or ETS alone were not significant predictors of cognitive development
The 182 of the WTC cohort versus Mt. Sinai births (Mt. Sinai Pregnancy Outcome Study) (Berkowitz et al., 2003)	Birth outcomes		No differences in mean gestational age, mean birth weight, frequency of preterm birth
			Twofold increase in IUGR among WTC exposed women
			No association found between probable PTSD and RR of preterm birth, LBW, or IUGR
The 52 of the 182 of the WTC cohort versus Mt. Sinai births (Mt. Sinai Pregnancy Outcome Study) (Engel et al., 2005)	Birth outcomes		PTSS and depression were associated with longer gestational duration
			PTSS was associated with decrements in head circumference
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2003)	Birth outcomes		Among African Americans, high PAH exposure was associated with lower birth weight ($P = .003$) and smaller head circumference ($P = .01$)
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2006)	3 years	BSID-II	Prenatal exposure to PAHs significantly associated with lower MDI at age 3, but not at 1 or 2 years. Prenatal PAH exposure was also unrelated to PDI
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2009)	5 years	WPPSI-R	High PAH levels, above the median of 2.26 ng/m^3, were associated with full-scale IQ (4.31 lower, $P = .009$) and verbal IQ (4.67 lower, $P = .002$) reductions compared to less exposed children
Dominican and African American (Washington Heights, Central Harlem, and South Bronx) (Perera et al., 2012)	6–7 years	CBCL	High PAH exposure, defined as greater than the median exposure from personal air monitoring, or detectable and higher maternal and cord adducts, was associated with symptoms of anxious/depressed and attention problems ($P \leq .05$)
Krakow, Poland cohort (2001–2006 enrollment) (Edwards et al., 2010)	5 years	RCPM	Children with higher than the median (17.96 ng/m^3) prenatal exposure to airborne PAHs had decreased RCPM scores at age 5. These reduced scores correspond to an estimated average decrease of 3.8 IQ points

BSID-II, Bayley-II Scales of Child Development; *CBCL*, Child Behavior Checklist; *ETS*, Environmental Tobacco Smoke; *GDS*, Gesell Development Schedules; *MDI*, Mental Development Index; *PDI*, Physical Development Index; *RCPM*, Raven Colored Progressive Matrices; *WPPSI-R*, Wechsler Preschool and Primary Scale of Intelligence-Revised. Included are studies related to the events of September, 11, 2001, as well as international studies of PAH exposures.

those not in the registry and who lived in a NYC census tract more than 5 miles from the WTC site, adjusted analyses found no differences in birth weight, gestational age of delivery, the odds of low birth weight, or the odds of preterm delivery. However, when comparing women with a high PTSD score to those with a lower score, increased odds of preterm delivery (AOR 2.48, 95% CI 1.05−5.84) and low birth weight (AOR 2.49, 95% CI 1.02−6.08) were detected (Lipkind et al., 2010).

In analysis of birth certificate data obtained from the Office of Vital Statistics in New York State and NYC, excluding lower Manhattan, Eskenazi et al. (2007) found an increased chance of very low birth weight (<1500 g) around the start of 2002 (i.e., among infants that would have been in their first or second trimester of gestation at the time of the September 11 attacks). This same finding was noted in births 33−36 weeks after the disaster (i.e., those conceived around or shortly after September 11). The authors suggest that these effects could be the result of the increased stress associated with a terrorist attack (Eskenazi et al., 2007). A study of maternal stress resulting from the 1998 ice storm in Quebec, Canada, found that more severe prenatal maternal stress was associated with decrements in Bayley Scales of Infant Development (BSID)−Mental Development Index (MDI) scores and parent-reported language abilities at 2 years of age (Laplante et al., 2004).

An interaction between exposure to PAHs and maternal stress has been suggested. Among children who experienced high prenatal exposure to PAH, maternal demoralization during pregnancy has a greater negative effect on children's neurobehavioral development. In the longitudinal study of 248 children from the Krakow cohort, maternal demoralization had an effect on syndromes of depression (anxious and withdrawn), rule-breaking and aggressive behaviors, and composite internalizing and externalizing scores at age 9 (Perera et al., 2003). These effects were seen only in those with high prenatal exposure. Both prenatal PAH exposure (Tang et al., 2014) and prenatal stress (Neeley et al., 2011) have been found to decrease hippocampal mature brain-derived neurotrophic factor (mBDNF) expression and signaling cascades for long-term potentiation (LTP), providing a potential mechanism for the observed interaction.

19.2.4 PAH-DNA adducts

PAH-DNA adducts provide a reflection of individual variation in exposure and toxicokinetics and have been associated with risk of cancer and reproductive and developmental effects (Tang et al., 2006; Perera et al., 2007). In the cohorts followed by researchers from the Columbia Center for Children's Environmental Health, mean DNA adduct concentrations in both maternal and fetal cord blood, as well as the proportion of samples with detectable adducts, increased across the populations studied in a manner consistent with the trend in estimated ambient exposure to PAHs ($P < .001$, northern Manhattan < WTC < Krakow < Tongliang). Data from these four populations indicate that the developing fetus may have a 10-fold greater susceptibility to DNA damage than mothers (Perera et al., 2005a).

Higher adduct levels have been associated with a number of adverse birth and neurocognitive developmental outcomes. In a cohort of 150 mother−infant dyads in Tongliang County, China, that received exposure from a seasonally operated, coal-fired power plant, those with higher than median PAH-DNA cord blood adducts weighed less than their low-exposure counterparts at 18, 24, and 30 months of age (Tang et al., 2006). Exposure for a longer duration was associated with shorter length at birth and height at 18, 24, and 30 months. In children who were in utero during power plant operation, there was a 91% increase in the odds of being developmentally delayed in the motor area for each 0.1-unit increase in cord blood adducts (0.1 adducts/10^8 nucleotides). The mean cord adduct levels in this cohort were 0.32 adducts/10^8 nucleotides (Perera et al., 2008). After the shutdown of the power plant, the head circumference of the children was greater than that of those born prior to the shutdown (33.766 vs 34.130 cm; $P < .05$), and consistent with the reduced levels of ambient PAHs and reduced levels of cord blood adducts (Tang et al., 2013).

In comparisons of these pre- and post-shutdown cohorts, it was also found that the mean level of mBDNF was significantly higher in the 2005 post-shutdown cohort (1266.56 vs 752.87 pg/mL; $P < .05$) (Tang et al., 2014). The brain-derived neurotrophic factor (BDNF) levels were inversely correlated with PAH-DNA adducts in cord blood in both cohorts ($P < .01$) and positively associated with average, motor area, and social area development quotients. Based on their analyses, Tang et al. (2014) deemed BDNF to be a potential mediator between PAH-DNA adducts measured in cord blood and later cognitive outcomes. This is consistent with findings that prenatal exposure to ETS, of which PAHs are a component, was linked to downregulation of BDNF through increased methylation of the BDNF exon (Toledo-Rodriguez et al., 2010).

Among women in the WTC cohort who were also exposed to ETS during pregnancy, increased PAH-DNA adducts were associated with reduced birth weight (276 g; 8% for a doubling of adducts) and head circumference (1.3 cm; 3% for a doubling of adducts) (Perera et al., 2005b). At age 3, children born to these mothers had an average reduction in MDI of 6% for a doubling of adducts (Perera et al., 2007). Lower MDI at age 3 was also found in the cohort of African-American and Dominican mothers and children (Perera et al., 2006).

Assessment of gene and environment interactions has found that both maternal and newborn haplotypes of CYP1A1 and CYP1B1 appear to be important effect modifiers of MDI at 12, 24, and 36 months of age (Wang et al., 2008, 2010). Subsequent analysis has identified a potential relationship between maternal haplotypes of XRCC1 and GSTM3, in addition to a maternal haplotype of CYP1B1, maternal PAH exposure, and newborn cord blood adducts in African Americans (Iyer et al., 2014). This study also found interactions with maternal PAH exposure and newborn cord blood adducts with newborn CYP1A2 and XRCC1 in African Americans, maternal XRCC1 among Dominican women, and newborn NQO1 in Dominican infants (Iyer et al., 2014).

19.2.5 Refinement of our susceptibility-exposure paradigm to assess the effects of in utero exposure to PAH aerosols on neurodevelopmental processes

The determination of the embryonic "critical period of development" for the brain structures involved in learning and memory processes in mice is based on the original work by Rodier (1976). This study identified the embryonic time frames for peak neurogenesis and neuroepithelial proliferation for cerebral cortex, hippocampus, septum, amygdala, corpus striatum, thalamus, hypothalamus, cerebellum, and olfactory bulb as the period from embryonic day E14 through E17. Rodier documented almost 40 years ago that the specific time of the central nervous system (CNS) insult is an important factor in subsequent effects on both anatomy and behavior. Therefore, this early work established what we refer to as the embryonic "critical period of development." The report suggested that the behavioral effects of toxicants such as benzo(a)pyrene (B(a)P) are similar in both rats and mice. This study was one of the first to demonstrate that mice could be used successfully as subjects in a variety of behavioral evaluation experiments.

Novel object discrimination testing has been used for over two decades because it is perfectly suited to test the effects of pharmacological and genetic interventions on learning and memory processes (Dere et al., 2007). When comparisons are made among experimental model systems of learning and memory, the object discrimination test is more closely related with the conditions under which human recognition memory is measured. This is primarily due to a shortened training period coupled with the fact that novel object discrimination does not induce high levels of arousal and stress in animals (Ennaceur and Delacour, 1988). The component of memory likely affected by PAH is the medial prefrontal cortex (mPFC) and is referred to as *relative recency memory* (Fuster, 2001). In support of this argument concerning relative recency memory, it is known that lesions to the mPFC impair relative recency discrimination in humans, nonhuman primates, and rodents across a wide range of stimulus modalities (Fuster, 2001). This is true despite the fact that, in some instances, recognition of novel and familiar stimuli is preserved.

Conversely, studies have reported that after bilateral mPFC lesions, an impairment or deficit is observed with respect to the ability to differentiate between previously presented and recently presented familiar objects (Mitchell and Laiacona, 1998). Results from our laboratory testing in utero of B(a)P aerosol-exposed $Cpr^{lox/lox}$ offspring in object discrimination paradigms were consistent with results from the aforementioned bilateral mPFC lesion studies. They support the suggestion that the mPFC is involved (at least in part) in making judgments regarding the sequence and order of object presentations. Additionally, the involvement of cortical glutamate receptors in performance of the novel object discrimination task in rodents is well established, as deficits and impairments have been reported following the application of AMPA (CNQX) or NMDA (AP-5) antagonists. By characterizing the effects of in utero exposure to PAH on $Cpr^{lox/lox}$ and brain-*Cpr*-null offspring, we further validate novel object discrimination phenotying as a measure of prefrontal and limbic circuit integrity by demonstrating that this behavior is negatively affected subsequent to in utero B(a)P exposure.

19.2.6 Refinement of our susceptibility-exposure paradigm to assess the effects of in utero exposure to PAH aerosols on behavioral phenotypes

Cytochrome-P_{450} is an important modifier of mental development at an early age. Recently, significant interactions were reported among a maternal haplotype in the cytochrome P_{450}1B1 (CYP1B1) gene (ACCGGC), PAH exposure, and reductions in the MDI in a cohort of children (Wang et al., 2010). These studies have important implications for our society; they are valuable from a translational standpoint because they can inform the design of molecular neurotoxicology studies using experimental model systems.

Recently, Xia et al. (2011) evaluated the effects of subchronic exposure to B(a)P (intraperitoneal injections of 6.25 mg/kg B(a)P/day for 14 weeks) versus diluent on both neurotransmitter receptor gene expression and performance in a Morris water maze. Microarray results revealed that 1016 genes were differentially expressed in B(a)P-treated specimens versus diluent controls. The Database for Annotation, Visualization, and Integrated

Discovery was used to analyze those genes differentially expressed in the gene ontology and Kyoto Encyclopedia of Genes and Genomes pathways. Their analysis showed that the most significantly affected category was behavior, and the fourth-highest was learning and memory. They ranked 22 genes involved in learning and memory and 25 genes associated with neuroactive ligand–receptor interactions. Both lists included upregulation of the ionotropic glutamate receptor *N*-methyl D-aspartate 2A (GRIN2A). A conclusion was that "neuroactive ligand–receptor interactions" were among the most negatively affected by B(a)P exposure at ($P < 7.7\ E-6$). In the Morris water maze test, B(a)P-treated rats had spatial learning deficits and had a decreased number of platform crossings and time spent in the target area, suggesting that B(a)P caused a deleterious effect on long-term memory. Several other studies have also suggested that neurotransmitter and neurotransmitter receptor gene expression play important roles in modulating neurobehavioral effects, especially within the context of learning and memory.

These results confirmed the notion that the PAH component can have a direct negative impact on the developmental expression of (1) key glutamatergic regulators of NMDA-mediated processes (receptor tyrosine kinase-MET) and (2) behavioral deficit phenotypes.

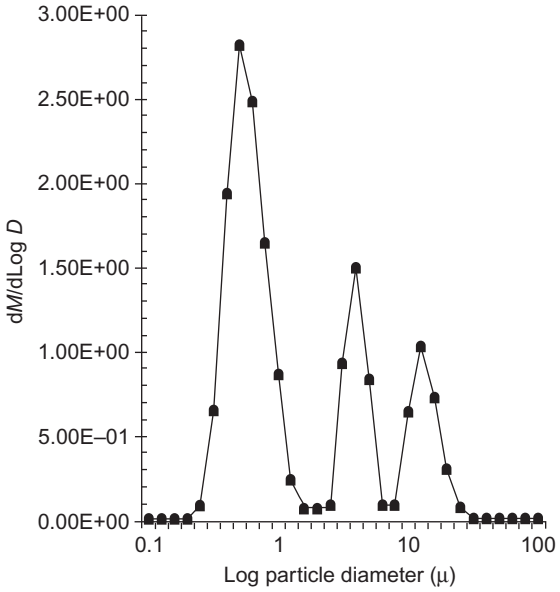

FIGURE 19.1 Differential particle-size distribution representative of B(a)P:carbon black aerosol delivered to timed pregnant $Cpr^{lox/lox}$ and brain-*Cpr*-null dams (100 μg/m^3).
During a typical exposure, aerosol was collected on substrates every 30 min during a 4 h exposure period from E14 to E17. Subsequent to the exposure period each day, substrate post-weights were uploaded into a custom impactor data reduction program to generate average particle-size distributions as previously described (Wu et al., 2003).

19.3 PAH experimental model systems

19.3.1 Toxicological observations from modeling B(a)P aerosols

In our experimental model system, $Cpr^{lox/lox}$ and brain-*Cpr*-null timed-pregnant dams were exposed to a B(a)P aerosol on E14–E17 exhibiting a trimodal distribution with a 93% cumulative mass less than 5.85 μm, 91% cumulative mass less than 10 μm, 57.6% cumulative mass less than 2.5 μm, and 43% less than 1 μm (Fig. 19.1 and Table 19.2). The characterization of the aerosol atmospheres generated in these studies was comparable to those generated previously for rat studies (Hood et al., 2000). The B(a)P aerosol used in our earlier rat studies comprised a mass median aerodynamic diameter with a geometric standard deviation of 0.9 ± 0.09 μm, compared to 1.0 ± 0.07 in the present *Cpr* mouse experiments. Analysis of live birth indices revealed no significant differences in the number of mouse pups born per litter between control $Cpr^{lox/lox}$ dams and B(a)P-exposed $Cpr^{lox/lox}$ dams. The analysis of the live birth index for control $Cpr^{lox/lox}$ dams was 6.12 ± 0.42, compared to 5.9 ± 0.9 for B(a)P-exposed $Cpr^{lox/lox}$ dams, and 5.7 ± 0.31, compared to 5.99 ± 0.41 for the control and B(a)P-exposed brain-*Cpr*-null dams, respectively. Statistical analysis ($P = .241$) indicated no significant difference in this index between these groups. This finding is consistent with our previous reports using rat and mouse models (Hood et al., 2000, 2006; Wormley et al., 2004a,b; Brown et al., 2007; Sheng et al., 2010). During the prenatal exposure period, as well as the subsequent preweaning period, there were no identifiable B(a)P-related effects on conventional reproductive indices of toxicity, and there were no convulsions, tremors, or abnormal movements noted in any of the control or B(a)P-exposed *Cpr* litters.

19.3.2 In situ generation of "oxidative metabolites" in neocortical tissue from in utero exposure to B(a)P aerosol

The quantitation of B(a)P metabolites from $Cpr^{lox/lox}$ and brain-*Cpr*-null offspring is shown in Fig. 19.2A and B. In neocortical tissue from in utero B(a)P aerosol-exposed $Cpr^{lox/lox}$ offspring, we found 4.5-, 7.8-, 9-, and 10-diols, 3.6- and 6.12-diones, and 3-OH and 9-OH B(a)P metabolites, indicating the presence of an active phase 1 biotransformation pathway (Fig. 19.2A). In contrast, in brain-*Cpr*-null offspring, all metabolites were essentially below the level of detection (note the change in the *y*-axis values in Fig. 19.2B). The important point in Fig. 19.2A is the identification of the developmental period over which there is formation and/or accumulation of the reactive 3-OH and 9-OH metabolites. These metabolites can

TABLE 19.2 Modeling of B(a)P: carbon black aerosols for *Cpr* studies.

Q_{tot} (slpm)	Q_{vap} (slpm)	Q_{hum} (slpm)	Q_{RBG} (lpm)	T_{mixch} (°F)	T_{vap} (°F)	$\rho(T_{vap})$ ($\mu g/m^3$)	T_{sat} (°F)	TCp (°F)	$T_{premix1}$ (°F)	$T_{particle2}$ (°F)	T_{mix} (°F)	Mixture $\rho(T_{mix})$ ($\mu g/m^3$)	Mixture CB(a)P at T_{mix} ($\mu g/m^3$)
10	2	7	1	132	234	3	192	276	270	79	132	5900	100
10	**2**	**7**	**1**	**114**	**234**	**3**	**192**	**288**	**281**	**80**	**135**	**7500**	**100**
10	2	7	1	104	234	3	192	295	288	80	137	8800	100
10	2	7	1	115	234	3	192	297	290	80	138	9100	100

TC_p and T_{premix} were generally high enough for top side of orifice plate $> T_{sat}$, rows 1–3 of each group of four rows. For each group, values greater than the second row are given in the fourth row for the purposes of evaluating overheating of the humidified B(a)P stream. Bold values in row 2 indicate the experimental conditions under which the studies were conducted. Assume $T_{Wright} = 70°F$; $m = 0.565$; $b = 37.432$ (for details, see Li et al., 2012).

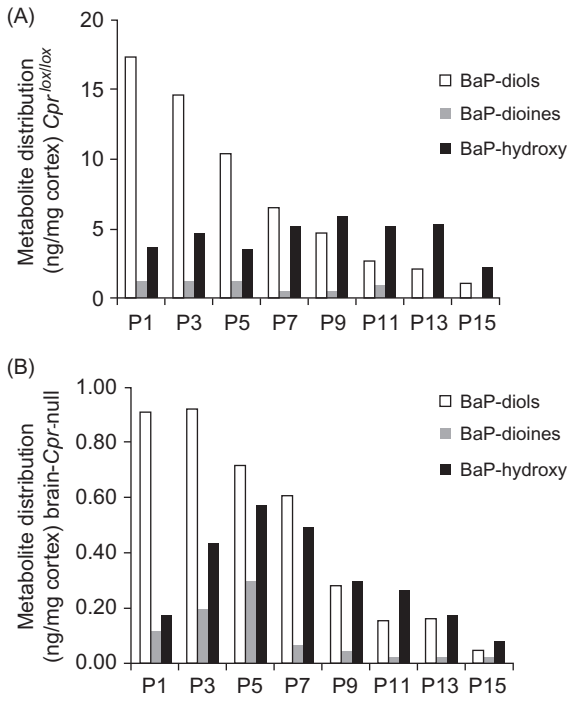

FIGURE 19.2 Metabolite distribution of cortical B(a)P metabolites during the critical postnatal period when synapses are forming for the first time in B(a)P-exposed *Cpr* offspring.
Timed-pregnant $Cpr^{lox/lox}$ or brain-*Cpr*-null dams received either carbon black only or 100 μg/m^3 B(a)P via nose-only inhalation on E14–E17 for 4 h/day. Offspring were sacrificed on P1, 3, 5, 7, 9, 11, 13, and P15. Shown are the distribution of metabolites detected in B(a)P-exposed (A) *Cprlox/lox* offspring or (B) brain-*Cpr*-null offspring.

further oxidize to form B(a)P quinones that can undergo redox cycling and generate reactive oxygen species (Li et al., 2012). F_2-isoprostane measures assessed the in vivo prefrontal cortex (PFC) oxidative milieu occurring primarily in neuronal membranes. Fig. 19.3 shows the quantitation of F_2-isoprostanes derived from neocortical homogenates from control and B(a)P-exposed $Cpr^{lox/lox}$ offspring (Fig. 19.3A), and control and B(a)P-exposed brain-*Cpr*-null offspring (Fig. 19.3B). (Note: There are no control *brain-Cpr-null* mouse isoprostane data for P3 to P15 due to the lack of available tissue.) The data document a sustained high neocortical tissue burden of F_2-isoprostanes in B(a)P-exposed $Cpr^{lox/lox}$, including the period from P7–P14, the extent of which was not observed in null mice (Fig. 19.3B).

19.3.3 Temporal modulation of NMDA-mediated developmental processes as a result of in utero exposure to B(a)P aerosol

Evidence supporting the hypothesis that an early insult to an NMDA-mediated signaling system leads to phenotypical changes that manifest later in life as elevated glutamate concentrations in mPFC is provided in Fig. 19.4A. Fig. 19.4A shows a time course plot indicating basal levels of glutamate in C57BL background controls, $Cpr^{lox/lox}$, B(a)P-exposed $Cpr^{lox/lox}$, and B(a)P-exposed brain-*Cpr*-null offspring obtained by microdialysis. Studies were carried out in a control group of mice performing the same task and B(a)P aerosol-exposed *Cpr* offspring over a 120-min sampling period. Quantitation of the results in Fig. 19.4 shows a statistically significant increase in the basal concentration of glutamate in the mPFC of B(a)P-exposed $Cpr^{lox/lox}$ offspring compared to WT C57BL, WT $Cpr^{lox/lox}$, or B(a)P aerosol-exposed brain-*Cpr*-null offspring. The basal glutamate concentrations obtained in our Cpr mouse model are in agreement with recent reports in the literature for dorsal hippocampus in a 129/SvEv mice KATII KO model (*45*) and for the PFC in a Wistar rat ethanol model (Chefer et al., 2011). These findings are consistent with the idea that increases in glutamate concentration promote altered inward currents via the NMDA receptor.

As a means of assessing the impact of in utero exposure to B(a)P aerosol on developmental NMDA-mediated processes, we evaluated developmental expression profiles for NMDA receptor subunits compared to those of Sp4 and Sp1 proteins with and without in utero B(a)P exposure. Inspection of the expression profile (P1–P15) in control $Cpr^{lox/lox}$ offspring (Fig. 19.5A, left panel) revealed that Sp4 expression is constitutively low on P1, reaches peak expression levels on P7, and subsides to constitutive levels by P15, which is consistent with earlier reports (Li and Pleasure, 2005). Conversely, the Sp4 developmental expression profile in B(a)P-exposed $Cpr^{lox/lox}$ offspring (Fig. 19.5B, right panel) indicates that as early as P1, Sp4 reaches near maximal levels and, by P3, it reaches peak expression levels and remains at maximal levels through P7. The interpretation of this finding is that the leftward shift in peak Sp4 expression is in response to in utero B(a)P aerosol exposure occurring during the E14–E17 period. The consequence of this exposure is the mistiming of peak Sp4 expression in B(a)P-exposed $Cpr^{lox/lox}$ offspring, which could significantly affect downstream signaling processes, perhaps in a way similar to those reported for Sp4 null mice (Zhou et al., 2007).

Having demonstrated that B(a)P exposure shifts the Sp4 expression profile to the left and given that NR2A is a Sp4 target gene, it is not surprising that B(a)P exposure also modulated the temporal expression of NR2A. The Sp4 nontarget NR2B developmental expression profile, on the other hand, for control $Cpr^{lox/lox}$ offspring (Fig. 19.5A, left panel) revealed that expression was low from P1–P3 and gradually increased until peak NR2B levels were present on P15. The NR2B developmental expression profile in B(a)P-exposed $Cpr^{lox/lox}$ offspring (Fig. 19.5B, right panel) is strikingly similar to that of

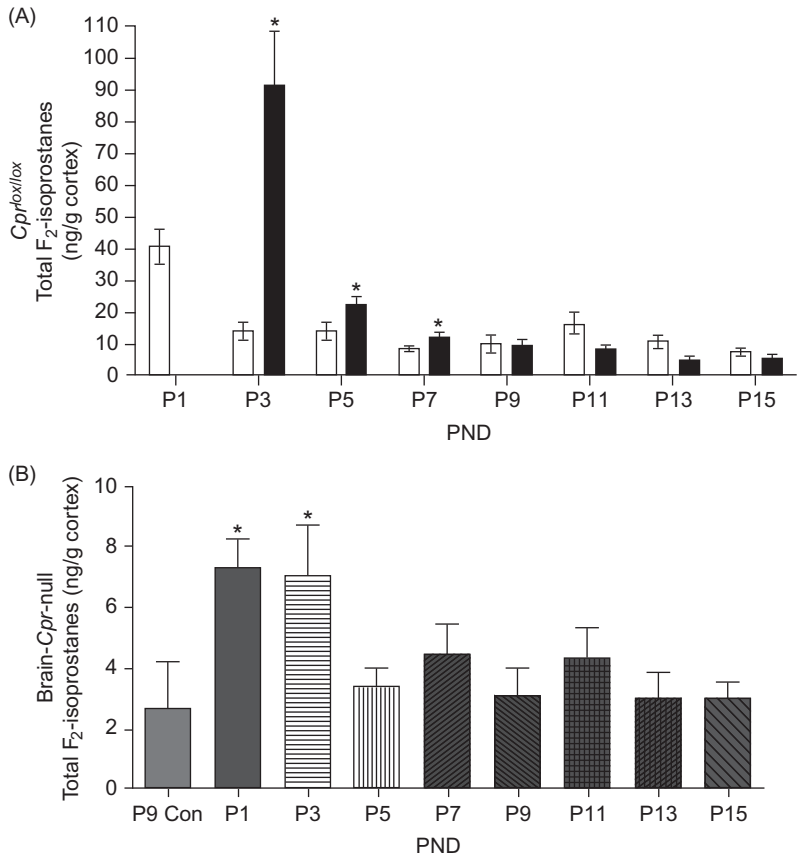

FIGURE 19.3 In utero exposure to B(a)P aerosol (100 μg/m^3) produces an approximate 10× higher concentration of F_2-isoprostanes on P3 in B(a)P-exposed $Cpr^{lox/lox}$ offspring as compared to B(a)P-exposed brain-*Cpr*-null offspring. (A) F_2-isoprostanes levels derived from control (white bars) and B(a)P-exposed $Cpr^{lox/lox}$ (black bars) offspring neocortex. (B) F_2-isoprostane levels derived from control PND9 (white bar) and B(a)P-exposed (black bars) brain-*Cpr*-null offspring neocortex. Due to the limited number of control offspring, only PND9 was used for time points in favor of reserving pups for behavioral determinations in later life. *$P = < .05$ versus control.

controls. What is apparent is the significant change in the NMDA subunit ratio of NR2B:NR2A on P7 in control $Cpr^{lox/lox}$ offspring (NR2B:NR2A = 1/0.4, as seen in Fig. 19.5A, lower-left panel) as compared to B(a)P-exposed $Cpr^{lox/lox}$ offspring (NR2B:NR2A = 1/0.78, seen in Fig. 19.5B, lower-right panel). Reminiscent of Sp4 expression, the NR2A developmental expression profile in B(a)P-exposed $Cpr^{lox/lox}$ offspring (right panel) is constitutively "on," beginning from P1−P5, and by P13, it reaches maximal expression levels compared to P15 in B(a)P-exposed offspring.

The developmental expression profiles for brain-*Cpr*-null offspring are shown in Fig. 19.6. Inspection of the expression profiles from these *null* mice (P1−P15) in control brain-*Cpr*-null offspring (Fig. 19.6A, left panel) revealed that Sp4 expression is constitutively low on P1 and reaches peak expression levels on P13. Conversely, the Sp4 developmental expression profile for B(a)P-exposed brain-*Cpr*-null offspring (Fig. 19.6B, right panel) indicates that as early as P1, Sp4 expression is evident, reaches maximal levels by P3, and then drops to constitutive expression levels by P7. What is apparent from the brain-*Cpr*-null offspring data is that the NMDA subunit ratio of NR2B:NR2A on early postnatal days (P1, P3, P5, and P7) in B(a)P-exposed brain-*Cpr*-null offspring (NR2B:NR2A = 1/0.5, seen in Fig. 19.6B, lower-right panel) is approximately the same as for P7 in control $Cpr^{lox/lox}$ offspring (NR2B:NR2A = 1/0.5, as seen in Fig. 19.6A, lower-left panel). Based on the results of a recent NR2A subunit knockout study in mice (Brigman et al., 2008), it would be reasonable to predict that in our null model, the absence of modulation in NMDA subunit ratios would mean that these mice would not exhibit a behavioral deficit phenotype. Such a finding would support our hypothesis that a significant deficit in behavioral learning results from the loss of proper temporal functioning of the NR2A subunit.

19.3.4 Rescue of spatial discrimination deficit phenotypes in brain-*Cpr*-null offspring subsequent to in utero exposure to B(a)P aerosol

Novel object discrimination testing was used to measure B(a)P-induced behavioral effects as described in our recent report (Sheng et al., 2010). Data in Fig. 19.7 show that control $Cpr^{lox/lox}$ offspring mice were better able to discriminate between novel and familiar objects (8.1 ± 0.31), as compared to B(a)P-exposed $Cpr^{lox/lox}$ offspring (2.0 ± 0.17). An analysis of variance revealed significant

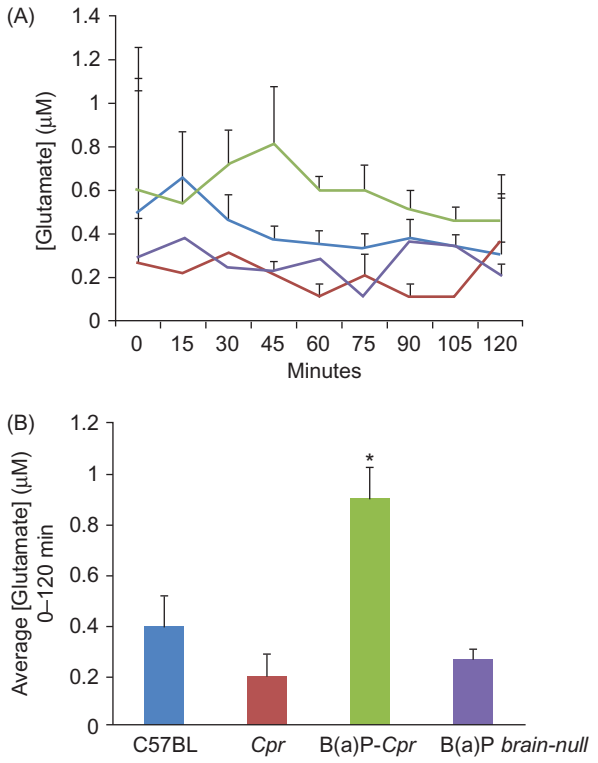

FIGURE 19.4 In utero exposure to B(a)P aerosol (100 μg/m^3) results in a robust increase in prefrontal cortical glutamate concentrations as assessed in awake behaving *Cpr*$^{lox/lox}$ offspring but not in brain-*Cpr*-null offspring (PND100). (A) Glutamate concentrations assessed from medial PFC as a function of time. (B) Quantitation of average glutamate concentrations over this time period (μM) assessed in control C57BL, control *Cpr*$^{lox/lox}$ offspring versus B(a)P-exposed *Cprlox/lox* and brain-*Cpr*-null offspring. See text for details (C57BL, blue; *Cpr*, red; and B(a)P-*Cpr*, green; B(a)P brain-*Cpr*-null, purple). *$P = < .05$ versus *Cpr*.

differences between the control *Cpr*$^{lox/lox}$ offspring (observational, small zone, and large zone) with regard to entries into the novel zone compared to the B(a)P-exposed *Cpr*$^{lox/lox}$ offspring. The exposed group exhibited a diminished ability to discriminate novel from familiar objects as indicated by a significantly reduced time as compared to controls ($P < .05$ for the 100 μg/m^3 group). Post hoc analysis using the Bonferroni's test revealed the significance at $P < .01$. Testing of control brain-*Cpr*-null offspring versus B(a)P-exposed brain-*Cpr*-null offspring in the 100 μg/m^3 group revealed no significant differences in object discrimination at 9.1 ± 1.4 versus 9.0 ± 0.8. Given that brain-*Cpr*-null offspring are incapable of producing significant levels of B(a)P metabolites via CYP1B1, these data strongly implicate a sustained-oxidative neocortical tissue metabolite burden during the critical period from P0 to P5 that contributes to the discrimination deficit phenotype observed in B(a)P aerosol-exposed *Cpr*$^{lox/lox}$ offspring.

Fig. 19.8 shows coronal sections of adult neocortical sections from *Cpr*$^{lox/lox}$ offspring that were analyzed for cytoarchitecture by immunohistochemical staining for NeuN. Neocortical neurons were counted in *Cpr*$^{lox/lox}$ offspring that had not been exposed to B(a)P in utero (A, low magnification; B, higher magnification) and compared to *Cpr*$^{lox/lox}$ offspring that were exposed to the B(a)P in utero (C, low magnification; D, higher magnification). Fig. 19.8E shows the plot resulting from stereological analysis of neocortical neurons subsequent to application of a two-tailed *t*-test. As can be seen, no significant differences were seen in the density of neocortical neurons as a result of in utero exposure to B(a)P aerosol.

Negative modulation of cortical inward currents in neurons derived from B(a)P-exposed *Cpr*$^{lox/lox}$ offspring. Finally, electrophysiology experiments were performed on ex vivo primary cortical neurons as a means of ascertaining potential B(a)P exposure-induced effects on cortical currents. On the seventh day in culture, ex vivo primary cortical neurons derived from control and B(a)P-exposed *Cpr*$^{lox/lox}$ offspring were voltage-clamped using the whole-cell configuration and held at −60 mV, in an Mg^{2+}-free external solution. The current−voltage (I−V) relationship of cortical neurons derived from control and B(a)P-exposed (100 μg/m^3) *Cpr*$^{lox/lox}$ offspring was nearly linear between −100 and −20 mV (Fig. 19.9A). Fig. 19.9B shows a representative current trace obtained from carbon black control and cortical neurons derived from 100 μg/m^3 B(a)P aerosol-exposed offspring at −80 mV using the same experimental configuration as in Fig. 19.8A. Although there were no apparent differences between the current recorded results in control and B(a)P-exposed cortical neurons at positive membrane potentials, there was a statistically significant, voltage-dependent decrease in the inward currents recorded at negative membrane potentials, as shown in Fig. 19.8C ($t = -2.92789$, $P < .0429$).

19.4 Implications

We have presented complementary biophysical, molecular, neurochemical, and neurobehavioral approaches examining how the PAH component of combustion processes negatively affects gestational and developmental processes in our experimental model systems. Our central findings are that in utero exposure to the PAH component of *c* (as B(a)P aerosol; Fig. 19.1) in the *Cpr* mouse results in a substantial neocortical oxidative tissue burden of B(a)P metabolites (Fig. 19.2) and F_2-isoprostanes (Fig. 19.3) during a period when synapses are developing for the first time. Such a sustained neocortical tissue burden might be expected to contribute to an increased cortical oxidative load. The presence of three distinct categories of metabolites (diols, diones, and hydroxy-derivatives) indicates an active phase 1 biotransformation pathway in our control *Cpr* mouse model. Present in B(a)

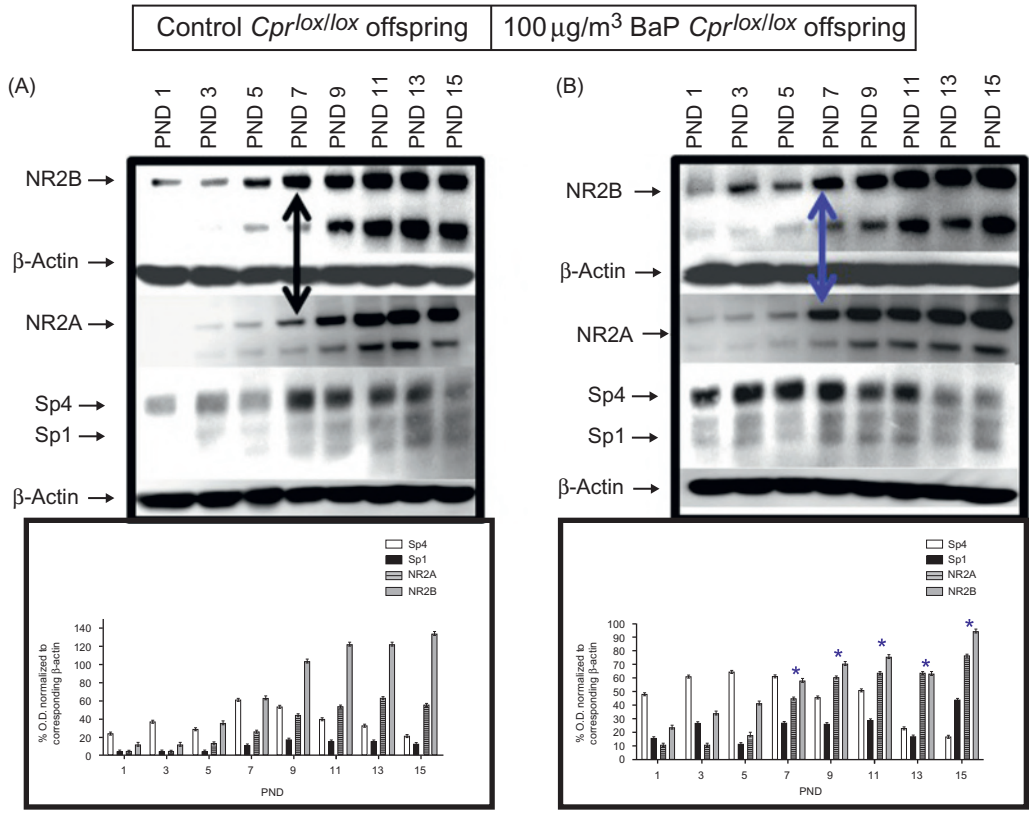

FIGURE 19.5 In utero B(a)P aerosol exposure (100 μg/m^3) induces a leftward shift in the peak temporal developmental expression of Sp4 and alters NMDA subunit ratios in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring (B, right panel) as compared to control *Cpr*$^{lox/lox}$ offspring (A, left panel). Representative results from a typical experiment. The panels show developmental expression profiles from PND1 to PND15 for NR2B-Sp4 nontarget gene, NR2A-Sp4 target gene, and Sp4-Sp1 proteins following SDS-PAGE of neocortical tissue from offspring gauged relative to internal β-actin controls. Lower panels represent quantitation of left and right upper panels. Post hoc analysis with the Bonferroni test with significance shown at $P < .05$.

P-exposed *Cpr*$^{lox/lox}$ offspring were the 4.5-, 7.8-, 9-, and 10-diols, the 3.6- and 6.12-diones and the 3-OH and 9-OH B(a)P metabolites. The important point in interpretation of these data is the identification of the developmental period over which there is formation and accumulation of 3-OH and 9-OH metabolites. These metabolites further oxidize to form B(a)P quinines that could undergo redox cycling and generate reactive oxygen species (Li et al., 2012). While the data from the brain-*Cpr*-null support the suggestion that accumulation of hydroxy-metabolites and their conversion into reactive intermediates in the *Cpr*$^{lox/lox}$ mouse likely contribute to the observed neurotoxicity, the levels may be below the threshold required to cause gross alterations in neuropathology (Fig. 19.8). Additional experiments will be needed to gain a better mechanistic understanding on why there were no observable pathologies in the neocortical cytoarchitecture in B(a)P-exposed animals. While speculative, one possibility may be that the plastic state of these young brains allows for certain compensations—for example, these mice may have an improved mitochondrial capacity. Such an effect has already been noted in response to severe hyperglycemia and hyperinsulinemia. Also, no studies are available on the effect of B(a)P on the NRF2 transcription factor, a master switch that regulates an antioxidant pathway. We may find that the susceptibility-exposure paradigm inherent to the present study causes upregulation of several or all antioxidant genes in response to Nrf2. Thus, despite dysregulation of glutamate homeostasis and ROS production, gross morphological alterations may be absent. Furthermore, it should be considered that the lack of change in total cell numbers would not take into consideration a more subtle change in cellular migration patterns and synaptogenesis in *Cpr*$^{lox/lox}$ offspring exposed in utero to a beno(a)pyrene aerosol.

The present study demonstrates that in utero exposure to a B(a)P aerosol in a *Cpr* experimental model system results in a temporal modification of upstream Sp4 transcription factor protein expression (Fig. 19.5) during a time when synapses are forming for the first time. It is likely that this response has negative effects downstream as follows: (1) Sp4 target gene−subunit ratio protein expression (NR2A, Fig. 19.5); (2) homeostatic glutamate

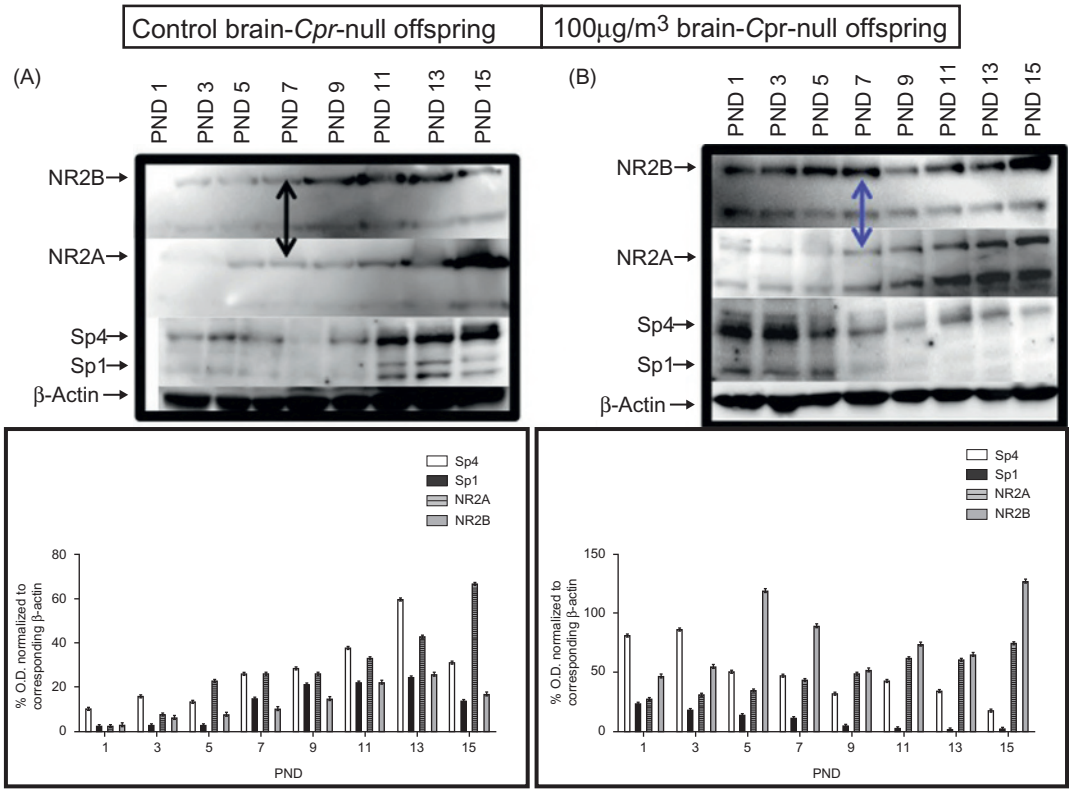

FIGURE 19.6 In utero B(a)P aerosol exposure (100 μg/m^3) upregulates developmental expression of Sp4 in brain-*Cpr*-null offspring (right panel) with accompanying dysregulation of NMDA subunit ratios as compared to control *Cpr*$^{lox/lox}$ offspring (left panel).
Representative results from a typical experiment. The panels show developmental expression profiles from PND1 to PND15 for NR2B-Sp4 nontarget gene, NR2A-Sp4 target gene, and Sp4-Sp1 proteins following SDS-PAGE of neocortical tissue from offspring gauged relative to internal β-actin controls. Lower panels represent quantitation of left and right upper panels. Post hoc analysis with the Bonferroni test with significance shown at $P < .05$.

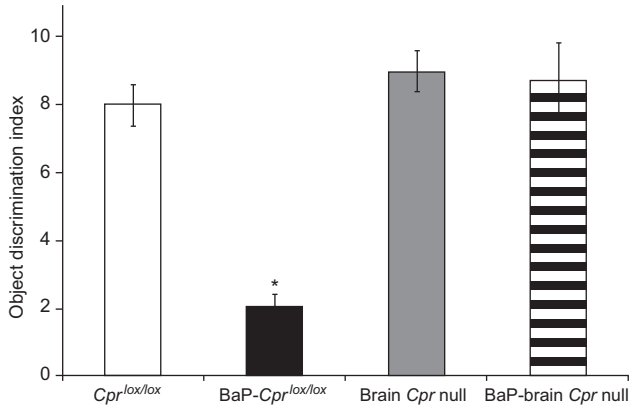

FIGURE 19.7 In utero B(a)P aerosol exposure induces a significant deficit in object discrimination in B(a)P-exposed *Cpr*$^{lox/lox}$ offspring as compared to control *Cpr*$^{lox/lox}$, WT brain-*Cpr*-null, or B(a)P-exposed brain-*Cpr*-null offspring.
Object discrimination index for control *Cpr*$^{lox/lox}$ offspring mice was 8.1 ± 0.31 and 2.0 ± 0.17 for B(a)P-exposed *Cpr*$^{lox/lox}$ offspring. An analysis of variance revealed a significant difference ($P < .05$ for the 100 μg/m^3 group). Testing of control brain-*Cpr*-null offspring revealed an index of 9.1 ± 1.4 versus 9.0 ± 0.8 for the B(a)P-exposed brain-*Cpr*-null offspring. Post hoc analysis with the Bonferroni test failed to reveal a statistically significance difference at $P < .05$.

neurotransmitter concentrations (Fig. 19.4); (3) novel object discrimination phenotype (Fig. 19.6); and (4) the magnitude of inward currents in cortical neurons (Fig. 19.9). The latter finding presents the potential for pharmacological augmentation of NR2A-mediated currents at cortical synapses as a means of modulating evoked activity in a structure-specific manner. The basal glutamate concentrations obtained in our *Cpr* experimental model system are identical to recent reports in the literature for dorsal hippocampus in a 129/SvEv mice KATII KO model and for the PFC in a Wistar rat ethanol model. These findings are consistent with the concept that increases in glutamate concentration promote alteration of inward currents via NMDA receptor expression. Whether these elevated concentrations of glutamate result from modulation in the activities of vesicular, glial, or astrocyte transporters (VGluT, GLT, or GLAST) remains to be seen.

That the mPFC integrity is, in part, important for the expression of object discrimination memory is supported by several studies where hippocampal lesions per se were found not to impair object discrimination (i.e., the ability to recognize a familiar object and discriminate it from a

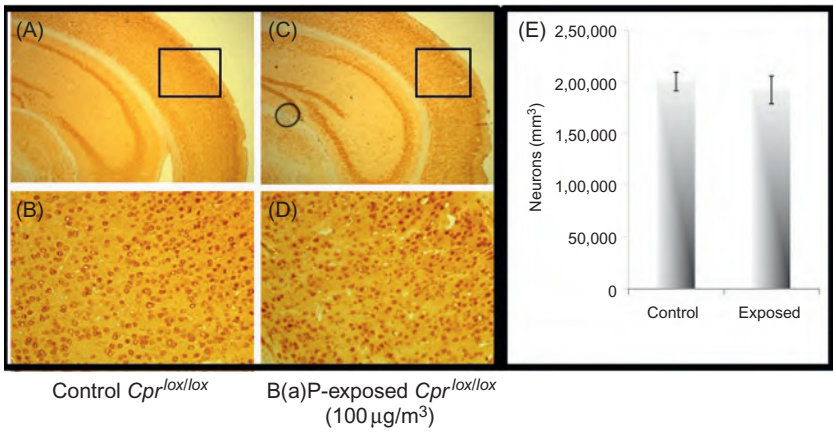

FIGURE 19.8 Post-behavior characterization of neocortical cytoarchitecture.
Coronal sections of adult neocortical sections from *Cpr*$^{lox/lox}$ offspring were analyzed for cytoarchitecture by immunohistochemical staining for NeuN. Neocortical neurons were counted in *Cpr*$^{lox/lox}$ offspring that had not been exposed to B(a)P in utero (A, low magnification; B, higher magnification) and compared to *Cpr*$^{lox/lox}$ offspring that were exposed to the B(a)P in utero (C, 4×—low magnification; D, 20×—high magnification). (E) It shows the plot resulting from stereological analysis of neocortical neurons subsequent to application of a two-tailed *t*-test. As can be seen, no significant differences in the density of neocortical neurons is observed as a result of in utero exposure to B(a)P aerosol.

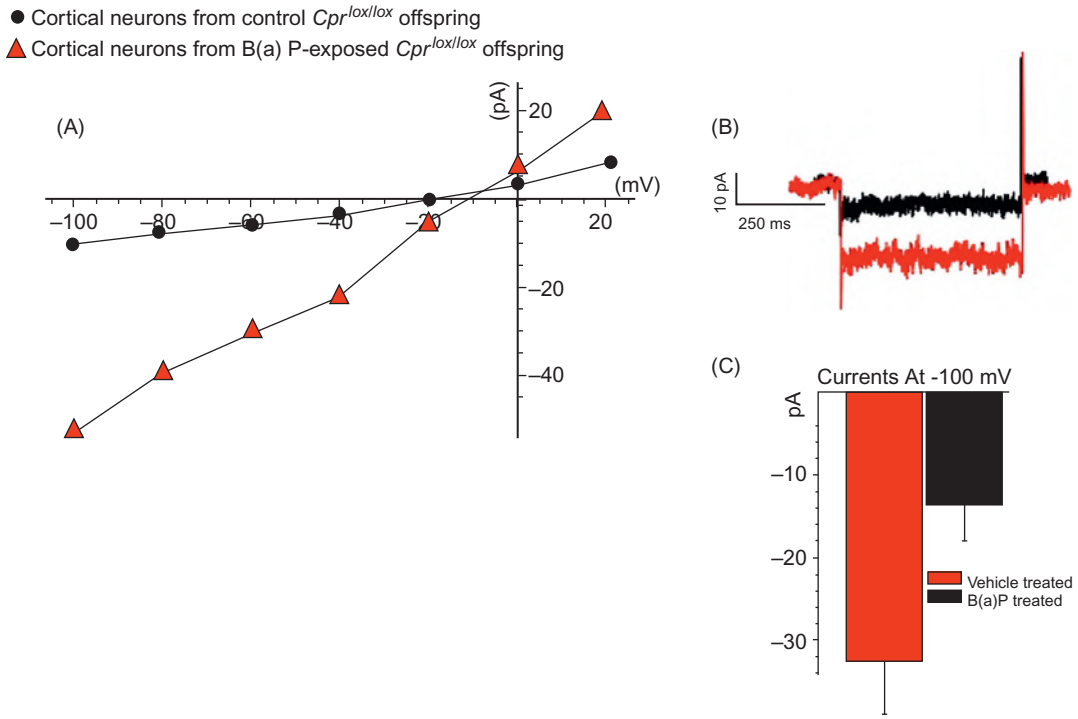

FIGURE 19.9 B(a)P aerosol exposure (100 μg/m^3) induces a voltage-dependent decrease in the inward currents of cortical neurons derived from B(a)P-exposed *Cpr*$^{lox/lox}$ offspring.
Cortical neurons were voltage clamped using whole-cell configuration in an Mg^{2+}-free external solution. Current–voltage relations were generated using a voltage step (1 s) protocol ranging from −80 to 20 mV separated by 20 mV from a holding potential of −60 mV. The experimenter was blind to the type of treatment. (A) Representative *I*–*V* currents for cortical neurons derived from carbon black and 100 μg/m^3 B(a)P aerosol-exposed *Cpr*$^{lox/lox}$ offspring. There is a voltage-dependent decrease in the magnitude of inward currents at negative membrane potentials in the cortical neurons derived from 100 μg/m^3 B(a)P-exposed *Cpr*$^{lox/lox}$ offspring. (B) Representative current traces obtained from carbon black control and cortical neurons derived from 100 μg/m^3 B(a)P aerosol-exposed offspring at −80 mV using the same experimental configuration as in (A). (C) Bar graph shows pA inward current recorded at −100 mV in control and B(a)P-exposed cortical neurons ($t = -2.92789$, $P < .0429$, $n = 3–5$).

novel object). These studies would suggest that the hippocampus is not totally required for the type of discrimination utilized in the present study. We agree that there are examples in the literature demonstrating that hippocampal lesions can impair object discrimination when the memory for the sample object includes spatial information. This is to say that when an object is presented in a complex environment with many visual and tactile cues, it appears less salient to the animal and therefore can be encoded by the hippocampus as part of this "complex" environment. Conversely, when an object is placed in an impoverished environment, it might appear highly salient and therefore can be encoded by the perirhinal cortex (for example), separate from the environment. The data from these studies may facilitate a discussion regarding the currently accepted hypotheses as to why rodents with hippocampal lesions may or may not be impaired in the object discrimination paradigm under complex but not impoverished environment conditions.

Other pharmacological studies have investigated the role of the parahippocampal glutamate receptor within the context of performance on the object discrimination task, and those results support our findings. The parahippocampal region is located dorsal to the hippocampal formation, and the evidence argues against a role of hippocampal NMDA receptors in object discrimination. In one study, intraseptal infusions of a low (0.4 mg) dose of the NMDA receptor antagonist AP-5 (d, l-2-amino-5-phosphono-pentanoic acid) improved object discrimination at a delay of 24 h, but not 45 min in rats when the dose was given either prior to or after the sample, or prior to the test trial. Another study reported that a subcutaneous (s.c.) injection of kynurenic or 5,7-dichlorokynurenic acid (antagonists at the glycine site of the NMDA receptor) given before the sample trial in doses of 0.6 or 30 mg/kg also improved one-trial object recognition in rats at a 1 h delay. These studies argue against a crucial role for the hippocampal NMDA receptor, thereby diminishing the necessity of performing in vivo microdialysis to delineate a role for glutamate in hippocampal formations within the context of performance of the object discrimination task.

It has been previously demonstrated that subacute exposure to B(a)P (0−200 mg/kg) (one intraperitoneal injection per day for 10 days) in adult mice modulated gene expression of NMDA-NR1 subunits in brain regions highly involved in cognitive function (Grova et al., 2007). Subacute exposure to B(a)P seemed to differentially affect NMDA-R1 expression in different parts of the brain. Cerebral regions, including the temporal cortex, showed no change in expression regardless of the B(a)P dose administered. In the hippocampus, exposure to B(a)P led to a 17-fold increase in a dose-dependent manner. In the frontal cortex, mRNA expression decreased 4−35 times with increasing doses of B(a)P. The results from these subacute studies in adult mice at relatively high doses in comparison to the present study suggest a link between in utero exposure to B(a)P aerosol, expression of functional obligatory NMDA-R1 mRNA, and impairments in short-term and spatial memory.

We know that postnatal brain development requires experience-dependent input that can induce the release of glutamate and thereby promote critical aspects of synaptic maturation. It is during this process of postnatal synaptogenesis that the effects of in utero B(a)P exposure on neural activity are most likely to alter the expression of genes, each with its unique temporal expression profile. In neurons of the neonatal brain, NR2A mRNA progressively increases during development and is dependent upon synaptic activity (Cull-Candy et al., 2001). In sensory pathways, Philpot et al. (2001) showed that the developmental shift from NR2B to NR2A can be postponed by sensory deprivation (Philpot et al., 2001). Studies such as these have predicted whether an alteration in the biophysical or molecular properties of NMDA receptors (NMDARs) from insertion of NR2A, loss of the NR2B, or both, places upper-limit constraints on the length of the critical period with respect to neural activity and experience-dependent fine-tuning of certain circuits.

A study by Zhou et al. (2007) reported on postnatal development of the hippocampus in the complete absence of the Sp4 gene. Notable observations were that the dentate granule cell precursors appeared to divide less during postnatal development in Sp4 *null* mice. Dentate granule cells from Sp4 *null* mice displayed less dendritic growth and arborization than those from wild-type mice placed into primary neuronal cultures. Additionally, Sp4 *null* mutant adult mice displayed both decreased neuronal cell density in the dentate granule layer and presented with a smaller dentate gyrus. The dentate gyrus is the primary gateway for hippocampal trisynaptic circuits to process the information received from the entorhinal cortex. The overall conclusion from this Sp4 *null* report is that abnormalities in circuitry exist in the *null* mouse characterized by a reduced hippocampal volume, representing a significant risk factor for some neurobehavioral deficit disorders. Collectively, results from genome-wide analyses predict an overlap of Sp protein transcription factor family members with the AhR network, and the Grin family of ionotropic glutamate receptor subunits (e.g., NR2A). Earlier studies have reported that knockdown of the transcription factor Sp4 in mice leads to increased numbers of highly branched dendrites during the maturation of cortical neurons in primary neuronal cultures (Ramos et al., 2007). The results from this report suggest that the Sp4 transcription factor likely controls dendritic patterning during development by limiting branch formation and by promoting activity-dependent pruning during a time when synapses are forming for the first time.

The signaling events that regulate deactivation kinetics for the establishment of fast synapses are preceded by changes in the subunit composition of the NMDA receptor at the synapse. This occurs during the postnatal critical switch period and is represented by the preweaning, postnatal period from P1 to P14. Results from a study by Brigman et al. (2008) illustrate the principle that NMDA receptor subunit proteins mediate certain forms of synaptic plasticity and learning. A touch-screen system was used to assess spatial discrimination learning in an NR2A subunit protein knockout (KO) mouse (Brigman et al., 2008). The study found that NR2A KO mice exhibited a significantly retarded discrimination learning pattern, supporting the currently accepted hypothesis that relative increases in the NR2A subunit protein during development ultimately serve to stabilize memories by constraining excessive synaptic plasticity.

Alternatively, NR2B-containing NMDARs appear to be the dominant form found during development, and their activity can initiate anatomical and functional plasticity, including LTP (Feldman and Knudsen, 1998). Paradoxically, NR2B does not contain GC-box elements within the 5′ promoter region, suggesting a potential mechanism for regulation of NR2A by the Sp4 transcription factor during the time when synapses are developing (Fig. 19.10). The rationale for the Sp4 transcription factor and its target genes as viable targets for B(a)P exposure-induced modulation during critical periods of development is based on the identification of canonical xenobiotic responsive element consensus sequences and GC-box elements within the 5′ promoter region (Fig. 19.11A). This is thought to render this gene and its target genes susceptible to modulation by B(a)P during critical phases of development. Fig. 19.11B shows the 5′ promoter region of the GRIN2B (NR2B) gene, which contains a single Sp4-binding site (GC box) and no xenobiotic response element (XRE) sequence in its 5′ promoter. The NR2B gene, however, does have seven XRE sequences in its 5′ UTR (Fig. 19.11B). Due to the absence of canonical XRE sequences and multiple GC-box elements in the 5′ promoter region, NR2B would not be classified as an Sp4 target gene. Conversely, the GRIN2A gene (NR2A) contains two Sp4-binding sites (GC-boxes) and six XRE sequences in its 5′ promoter (three in a forward orientation and three in a reverse orientation) (Fig. 19.11C). There are two additional XRE sequences in the ORF of GRIN2A (one forward and one reverse), as is shown in Fig. 19.11C. The NR2A receptor subunit thus qualifies as a Sp4 target gene and has been reported as such (Liu et al., 2003).

Clearly, the novel object discrimination task is sensitive enough to detect deficits in response to object discrimination that are reflective of learning and memory impairments. PAH aerosol-induced negative modulation in the temporal developmental expression of Sp4 demonstrates a particular sensitivity to environmental exposure during a critical period of development. Studies in the immediate future will seek to elucidate the functional

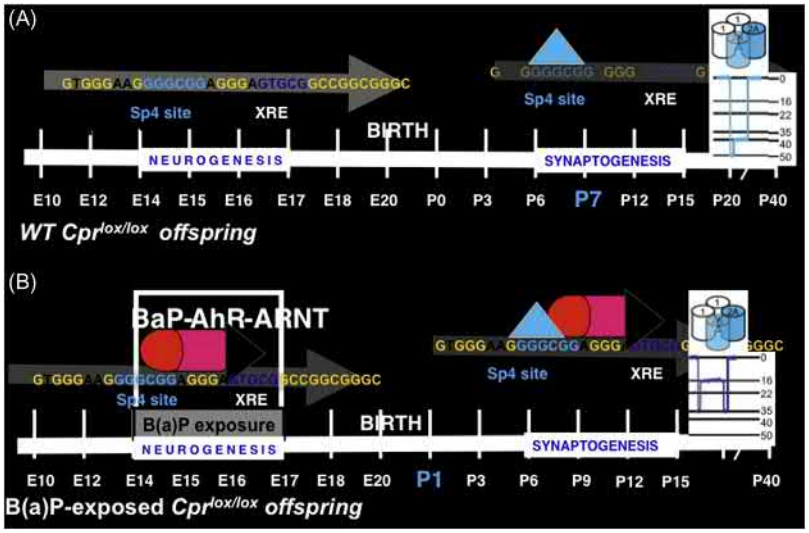

FIGURE 19.10 (A) Normal glutamate and NMDA-NR2A homeostasis. (B) Dysregulated (elevated) cortical glutamate and NMDA-NR2A homeostasis. (A) Normal temporal activation of Sp4 expression and of its target genes during embryonic development in timed-pregnant control $Cpr^{lox/lox}$ dams is depicted from E10 to E20 (birth). During the early postnatal period, the Sp4 target gene NR2A facilitates in establishing constitutive NMDA-NR2A-driven cortical currents. This occurs during the *critical period of synapse formation* (P7−P15) and contributes to a normal object recognition/discrimination phenotype in $Cpr^{lox/lox}$ offspring. (B) Premature activation of Sp4 expression and of its target gene, NR2A, occurs subsequent to in utero exposure of timed-pregnant $Cpr^{lox/lox}$ dams to B(a)P in aerosol. During the early postnatal period in exposed $Cpr^{lox/lox}$ offspring, dysregulated Sp4 and target gene expression results in upregulated NMDA-NR2A-driven cortical currents. This occurs during the *critical period of synapse formation* when NR2A-NR2B subunit ratios are altered (P7−P15), thus contributing to an impaired object recognition/discrimination phenotype in exposed $Cpr^{lox/lox}$ offspring.

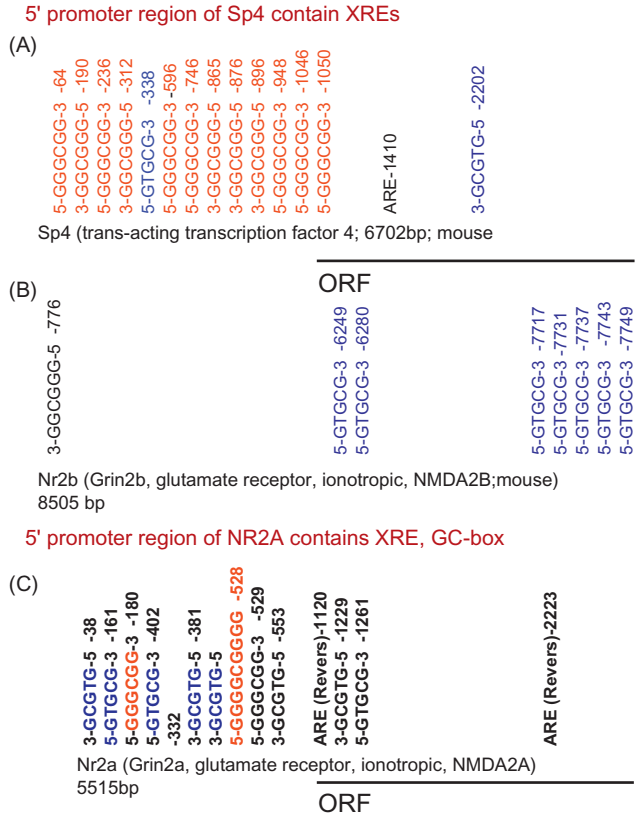

FIGURE 19.11 Promoter analyses for canonical XRE and GC-box consensus sequence requirements in Sp4 target gene versus nontarget genes.
(A) Core Sp4 promoter; (B) core GRIN2B (NR2B) promoter; (C) core GRIN2A (NR2A) promoter. *Blue*—XRE, xenobiotic response element; *Red*—GC box/Sp4-binding site; *Black*—ARE, antioxidant response element.

changes and mechanisms undergirding alterations in Sp4 target gene-driven neural activity (NMDA-NR2A-mediated) and plasticity using our *Cpr*-null model. Translating these new concepts into animal studies offers the promise of advancing our ability to establish the presently absent mechanistic connections between exposure-induced diseased phenotypes, due to disturbances in temporal expression patterns during critical windows of development. In order for significant advances in the field to come to fruition, molecular-level hypotheses of in utero air pollution exposure effects on later-life phenotypes must continue to be investigated by multidisciplinary teams.

19.5 Other model systems used for PAH-induced neurotoxicity and role of the microbiome

The aforementioned narrative details the sequelae of events subsequent to prenatal and postnatal exposures to PAHs through aerosol as the emphasis of this chapter is on neurotoxicity of agents released during chemical warfare and related operations that involve combustion during war settings. However, some rodent, nonrodent, and human cell line model systems have been used that studied the impact of PAH-induced neurotoxicities subsequent to noninhalation exposures. Some of those studies are briefly mentioned here as the research strategies used and the mechanisms identified will be of relevance to aerosol exposures as well.

Male Wistar rat pups, which received 2 μg B(a)P/kg bw on PND5 through intracisternal administration revealed neurobehavioral (spontaneous motor hyperactivity) changes, and alleviation of dopamine in striatum and neurodegenerative (histopathological changes in striatum) consequences in adult life (Das et al., 2016). The role of NMDAR in regulating inhaled B(a)P toxicity is mentioned elsewhere in this chapter. Additional evidence in support of NMDAR perturbation leading to neurotoxicity induced by orally administered B(a)P was furnished by Chepelev et al. (2016). Microarray profiles of adult male Muta™ mouse orally administered with 1, 35, or 70 μg B(a)P/kgbw showed increased expression of NMDAR subunits Grina and Grin2a. The B(a)P-induced transcriptional profiles of hippocampus from Muta mouse were similar to the neurotoxicity outcomes seen in B(a)P-exposed rats and mice.

Metabolomic studies also provided compelling evidence that postnatal subchronic oral B(a)P exposures (2 mg/kg/day for 7 weeks) contribute to neurobehavioral impairment in Sprague−Dawley rats (Li et al., 2018). Pathway analysis revealed that those affected were energy, methionine, cysteine, and glutathione metabolic pathways, which were correlated to cerebellum injury. Using the above-mentioned experimental regimen, in addition to metabolomics, epigenetic and transcriptomic studies were conducted by this research group (Wang et al., 2018) to determine the mechanistic basis of B(a)P neurotoxicity. Their studies revealed that exposure to B(a)P caused changes in the levels of DNA methylation and expression profiles of ncRNAs and mRNAs. Additionally, changes in metabolism-related enzymes that regulate the synthesis of glycine, phenylalanine, and leucine were observed in the hippocampus. Taken together, these results suggest that long-term exposure to B(a)P leads to depletion of energy pools (which are key to meeting the energy requirements of neurons) and disruption of neurotransmitter homeostasis, ultimately leading to cognitive dysfunction.

Aside from rodent models, developmental exposure to B(a)P has been shown to affect larval behavior and adult learning in zebrafish (Knecht et al., 2017a). Experiments carried out by this research group (Knecht et al., 2017b) also revealed the transgenerational impact of the above-mentioned deficits as manifested by alterations in morphological (increased body mass index in F2 generation),

physiological (decreased heart beat and mitochondrial function in F0 and F2 generation; increased oxygen consumption in F2 generation), and neurobehavioral parameters (social-anxiety-like behavior in F0 generation; hyperavoidance behavior in F2 generation).

Another study using cell systems lends support to the notion that early life exposures to PAHs may have health effects later in life. Slotkin et al. (2017) used in vitro models to demonstrate the effect of developmental PAH exposure on neurotoxicity. This group used two cell types, which shows differences in "decision node" or period of neurodifferentiation due to PAH exposure. The two cell models used were the Neural Stem Cell (NSC) model, which represents differentiation of stem cells into neuronal or glia cells and the PC12 model, which represents multiple processes that include neuronal cell growth, extension of neuritic projections, etc. The PAHs evaluated were either single compounds [B(a)P] or an environmentally derived PAH mixture [Elizabeth River Sediment Extract (ERSE); from a Superfund site]. Benzo(a)pyrene had a profound effect on the PC12 cells, impacting the transition from cell replication to cell growth. On the other hand, ERSE affected the NSC model resulting in increased cell replication and enhanced neurodifferentiation. These studies bring into light two important aspects: (1) the chemical behavior and the consequent toxicity differs among individual PAH compounds and; (2) the potential window of neurodevelopmental susceptibility is greater than thought previously.

Recently there has been a surge in literature on the role played by the microbiome in maintaining optimal health as well as the susceptibility to disease. How the microbiome affects the xenobiotic metabolism through the host hepatic biotransformation enzymes and impacts human health has not been well researched (Clarke et al., 2019). On one hand, the enzymes from microbes that reside in the host may generate neurotoxic metabolites of their own in the host's body, in addition to those arising from their own metabolism of xenobiotics. Gut microbes were known to stimulate neurons of the enteric system, which sends signals to the brain through the vagus nerve. As a consequence, the hypothalamus−pituitary−adrenal axis is affected, resulting in memory and cognition disorders (Galland, 2014). Dietary intervention through increased intake of nutrients/antioxidation, pro- and prebiotics is one strategy to offset the damage to the CNS caused by these microbes. Whether this strategy would work for all neurotoxicants and/or neurotoxicants that are highly lipophilic and sequestered in the CNS is not known. Additionally, to what extent this strategy would be effective for long-term developmental exposures to airborne xenobiotics is open for speculation. There is a lot of scope for omics studies to delineate the neurotoxicity of PAHs and other semivolatile xenobiotics and also characterizing the resident microbiome and its contribution to either ameliorating or exacerbating the toxicity of these compounds.

19.6 Concluding remarks and future directions

The research described in this chapter represents the contribution toward leading the development of a novel methodology to systematically discover and develop therapeutic glutamate receptor/aryl hydrocarbon receptor antagonists that will mitigate the neurotoxicity associated with in utero exposures to B(a)P aerosol. A fundamental premise of future research takes into account the need for simultaneous temporal measurements, and integrative physiological analysis of critical signaling processes, to better understand the mechanisms of PAH exposure-induced neurotoxicity. Future experiments should also define the signatures of PAH exposure-induced neurotoxicity and novel therapeutics/protectants in experimental model systems. Additionally, monitoring changes in the microbiome profile would help trace the "neurodevelopmental fingerprints" for single PAH compounds and mixtures in the presence or absence of specific microbial species.

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Chapter 20

Thallium

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20.1 Introduction

Thallium (Tl) is a soft, bluish-white metal that occurs naturally in the Earth's crust. It was discovered by Sir William Crookes in 1861 while making spectroscopic determinations of tellurium in residue material from a sulfuric acid plant. The name comes from the Greek word "thallos," which means a green shoot or twig, a reference to the green spectral emission lines originally used to identify the element. It is a heavy metal (density 11.83 g/cm^3, atomic number 81) whose mainly use is in the electronics industry (e.g., infrared detectors, semiconductor materials) with smaller quantities used in glass manufacturing and pharmaceutical industries, including the radioactive isotope Tl-201. Thallium can be released into the environment by cement manufacture, the burning of certain coal deposits, and the production of nonferrous metals (Kazantzis, 2000; Peter and Viraraghavan, 2005). Normally, Tl is found in the environment in low concentrations, commonly as the Tl (+1) or Tl (+3) oxidation state. The MCL (maximum contaminant level) for Tl in drinking water is 0.002 mg/L (US EPA, 2009a), the level at which no adverse human health effects are anticipated. Commercial Tl is usually obtained during the refining process for iron, cadmium, lead, or zinc (USGS, 2019). Thallium is a highly toxic element and salts of Tl are colorless, odorless, and tasteless. Thallium has no known biological function and has been the least studied of the toxic metals including lead, mercury, and cadmium. Thallium salts were introduced as pesticides in Germany in 1920. The sulfate salt is most commonly used and has been widely used as a rodenticide and ant killer. Tl has been associated with intentional and accidental poisonings since that time, although problems decreased greatly after its use was banned in many parts of the world (Saddique and Peterson, 1983).

20.2 Background

Thallium has two important oxidation states, Tl (+1) and Tl (+3). The trivalent form more closely resembles aluminum and the monovalent form more resembles alkali metals such as potassium. The toxic nature of the monovalent Tl is due to its similarity to potassium in ionic radius and electrical charge (US EPA, 2009b). Thallium sulfate use as a pesticide was restricted in 1965 in the United States and the World Health Organization (WHO) recommended in 1973 against its use as a rodenticide due to its toxicity (WHO, 1973). From 1912 to 1930, thallium compounds were used extensively for medicinal purposes, for example, in the treatment of ringworm (because of the depilatory effects), dysentery, and tuberculosis. The narrow margin between toxicity and therapeutic benefit, however, eventually eliminated the practical use of these compounds. Due to its highly toxic nature, delayed symptoms, and lack of taste or odor, Tl salts have been used in suicide attempts and in the intentional poisoning of individuals or small groups of people. Although the reported symptoms of Tl poisoning are diverse, the classic syndrome involves gastroenteritis, polyneuropathy, and alopecia. Fictional accounts of Tl as the agent of an intentional poisoning include Agatha Christie's book, *The Pale Horse*. More recent accounts or suspicions of Tl use include medical case reports as well as lay press reports. Chronic Tl exposure has been reported in industrial settings and exposure limits have been established (Peter and Viraraghavan, 2005). However, there are currently insufficient epidemiological data concerning the chronic effects of Tl on humans, as well as a lack of data concerning the mutagenic effects and the effect of Tl on genetic material (Rodríguez-Mercado and Altamirano-Lozano, 2013). The radioactive isotope Tl-201 is a gamma emitter and is used in cardiac imaging, similar to technetium-99, with a

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half-life of approximately 3 days. Although Tl-201 is the most common isotope in use, Tl-204 decays by beta particle emission and has a half-life of 3.8 years.

20.3 Toxicokinetics

Thallium is rapidly absorbed from the gastrointestinal tract and is well absorbed through the skin. There is little information concerning absorption from the respiratory tract. Once absorbed, Tl is rapidly distributed throughout the body to all organs, with the highest concentrations occurring in the kidney following an acute exposure. Both monovalent and trivalent Tl appear to distribute in similar manners, and it is not known if metabolic processes can change the valence state. Thallium can pass through the placental barrier as well as the blood–brain barrier (Sullivan, 1992).

Elimination of Tl is mainly through the gastrointestinal tract but elimination also occurs through the kidneys, saliva, hair, skin, sweat, and breast milk. Relative amounts excreted by each route vary by species. Thallium is likely excreted through intestinal and gastric secretions associated with potassium loss or excretion. Likewise, reabsorption of Tl also occurs, mainly from the colon. The estimated biological half-life of Tl is 10 days but values up to 1 month have been reported (WHO, 1996).

20.4 Mechanism of action

Although the exact mechanism of action of Tl is unknown, its similarity to potassium has been shown to play a significant role. Thallium has an atomic radius similar to potassium and has shown a 10-fold affinity over potassium in Na^+/K^+-ATPase, resulting in lower activity of the enzyme. Tl inhibits the influx and efflux of potassium in mitochondria, without affecting the movement of sodium. In addition to disturbing mitochondrial function, Tl has been shown to increase the levels of hydrogen peroxide and also to increase lipid peroxidation and oxidative stress (Hanzel and Verstraeten, 2005). The metabolism of glutathione can be disrupted by Tl, increasing the susceptibility to reactive oxygen species (Cvjetko et al., 2010). Thallium can also inactivate sulfhydryl groups, including those affecting the permeability of the outer mitochondrial membrane. Thallium can act as a Lewis acid, having an affinity for organosulfur compounds, which may account for its action to cause hair loss. The binding of cysteine by Tl may inhibit keratinization of hair by preventing the cross-linking of proteins (Mulkey and Oehme, 1993).

20.5 Toxicity

The available human literature on Tl is mainly case reports from the results of acute poisonings, accidental ingestions, or suicide attempts. Although the acute classic syndrome of Tl poisoning involves gastroenteritis, polyneuropathy, and alopecia, not all these effects are observed in every case. The onset and sequence of symptoms will vary with the dose and duration of exposure. The lowest known toxic dose in a human is 0.31 g, which was reported to cause symptoms but did not cause death (Cavanagh et al., 1974). Oral doses of 6–40 mg/kg have been lethal within 10–12 days. Other oral human toxic doses are given as 10–15 mg/kg (WHO, 1996; Moore et al., 1993). Children have been poisoned with Tl at 4–8 mg/kg.

Several hours following an acute exposure, the initial symptoms may include gastroenteritis including nausea, vomiting, and diarrhea. With a relatively small dose, these symptoms may be relatively mild and diffuse, with little progression for 2–5 days. Gastrointestinal bleeding or constipation may then develop along with central and peripheral nervous system effects. These include paresthesia with reports of the feeling of "burning feet." Additional neurological symptoms can include lethargy, delirium, seizures, and coma (Tsai et al., 2006). An initial presentation simulating Guillain-Barré syndrome has been reported (Misra et al., 2003). Nonspecific kidney and liver damage can develop. In severe exposures, circulatory symptoms may include hypertension, tachycardia, and cardiac failure. Initial dermatological involvement may include anhydrosis (which can cause fever), and this can be followed some time later by diaphoresis. In the second week following exposure additional dermatologic symptoms appear, including increased darkening of the hair papillae followed in several days by a developing alopecia. By 3 weeks following exposure there may be almost complete alopecia. At this time in the syndrome there may be ataxia and tremors with a painful neuritis in the lower extremities that may be severe. Following a lethal dose, death commonly occurs within 10–12 days caused by renal or cardiac failure. Recovery from Tl poisoning can require several months and residual neurological problems may remain including weakness, memory impairment, and psychological disturbances (Pau, 2000; Tsai et al., 2006).

20.6 Risk assessment

Although reported as an agent of intentional poisoning for an individual or small group of people, the broad use of Tl as an agent in chemical warfare or terrorism has not

been reported (Salem et al., 2008). The most commonly available radioisotope of Tl-201 is a gamma emitter with a short half-life, making it a poor candidate for a radiological dispersion device (Burnham and Franco, 2005; Chin, 2007). Although the Tl-204 isotope is a beta emitter with a half-life of 3.8 years, its commercial use is limited. The chelating agent used for treatment of Tl exposure (Prussian blue) is also used in the treatment of radiocesium exposure, thus it is included in many antidote stockpiles (Ansari, 2004).

20.7 Treatment

Diagnosis of Tl poisoning is based upon exposure history if available, compatible clinical time course and symptoms, along with the finding of above-background levels of Tl in urine, serum, or other clinical specimens. The appropriate methodology for urine or blood Tl includes atomic absorption spectroscopy (flame or flameless) as well as other methodology such as ICP (inductively coupled plasma emission spectroscopy). Use of colorimetric analyses of these specimens can lead to false positives (CDC, 1987). Normal or background concentrations of Tl in urine are given as <0.5 μg/L up to a level of <10 μg/L, depending upon the laboratory reference, with concentrations elevating several hundred- to several thousand-fold following an acute exposure. Treatment should be initiated when 24-h urinary Tl excretion exceeds 0.5 mg. Additionally, a toxic level of Tl in the urine of >300 μg/L has been suggested (Sullivan, 1992). Blood Tl levels in exposure situations are less well characterized and values above 5 μg/L are considered to be evidence of excess Tl exposure (CDC, 1987). Prussian blue [ferric-hexacyanoferrate (II)] is the treatment of choice for Tl exposure in that it acts by binding Tl in the gastrointestinal tract, making it unavailable for reabsorption (Altagracia-Martínez et al., 2012). This will increase the fecal excretion of Tl and decrease the half-life. Prussian blue is also used in the treatment of radiocesium and acts by the same mechanism (Yang et al., 2008). The suggested dosage regimen is 3 g given orally three times a day for adults and adolescents. Children between the ages of 2 and 12 years can be given 1 g orally three times a day. The clinician should insure proper gut functioning because constipation is a common finding in Tl intoxication. Fluid diuresis and other symptomatic and supportive care should also be provided. Recently a workgroup composed of international experts assembled to review and provide recommendations on the use of extracorporeal treatment for poisonings. In the case of severe Tl poisoning, the workgroup strongly recommended extracorporeal removal for Tl, although it was acknowledged that limited evidence exists (Ghannoum et al., 2012).

20.8 Concluding remarks and future directions

Thallium remains a toxic metal of concern for both accidental and intentional exposure. Its historical use as an intentional poison against individuals or small groups is based on its delayed onset of symptoms and the nature of its salts being tasteless and odorless. Although the incidence of intoxication with Tl has been decreasing with its decreasing availability, clinicians should remain familiar with its clinical features, diagnostic considerations, and treatment regimens. History has shown Tl not to be an agent of choice for chemical warfare or terrorism, and the use of radiological Tl for these purposes is also unlikely. However, preparations for such an unlikely event are relatively straightforward with major considerations being the stockpiling of Prussian blue, also needed for radiocesium treatment, and the retention of analytical capabilities for Tl in biological and other samples.

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Chapter 21

Arsenicals: toxicity, their use as chemical warfare agents, and possible remedial measures

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21.1 Introduction

Arsenic is a metalloid (semimetal) member of group V elements of the periodic table, having oxidation states of +3 (As III) and +5 (As V). Both species occur in organic as well as inorganic compounds. Arsenic combines chemically with most nonmetals to form a variety of both inorganic and organic compounds (Susan et al., 2019). Arsenic-based chemical agents have revealed long-term effects in humans that can be life threatening if left untreated. A number of organic arsenicals have been developed for use as chemical warfare (CW) agents (Radke et al., 2014). The use of chemical weapons in war guarantees overall efficiency because chemical agents do not depend on explosive force to achieve objectives, while infrastructure remains intact. Also, the production, storage, and transport (in a sealed container) require relatively low cost and low technology. The use of chemical agents, including arsenical agents, offers many unique advantages, which vastly differ from those of conventional weapons and are of human and ecotoxicologic relevance (Polat et al., 2018). The story of arsine and lewisite encapsulates the key elements of the history of chemical weapons and their continuing influence. Although information about arsenic and its inorganic and organic derivatives is well documented, there is very little literature available on their role as CW agents. This chapter provides readers with updated information about the organic arsenicals as CW agents and also gives a comprehensive account of toxicity due to inorganic arsenicals. Unfortunately, chronic arsenic poisoning due to exposure to agricultural agents currently remains a significant cause of morbidity and mortality, particularly in the developing world. Inorganic salts of arsenic are the most toxic, in particular trivalent arsenic (III) such as arsenic trioxide, and pentavalent arsenic (V) which is approximately 60 times less toxic. Arsenic (III) has many toxic effects relating to enzyme inhibition, but in particular it inhibits pyruvate dehydrogenase, interfering with the Kreb's cycle, whereas arsenic (V) directly poisons ATP by displacing inorganic phosphate and other features may result from binding sulfhydryl groups of surface proteins and oxidative stress from glutathione depletion. The lethal oral dose in humans is variously reported as 120−200, 100−300, or 200−300 mg (Rosenberg et al., 2016).

21.2 Background

CW agents are incapacitating, damaging, toxic, and lethal compounds. It is known that CW agents have been used since 600 BCE. Even though these agents have been used many times intermittently, their use increased significantly in the course of World War I (WWI), when cyanide, phosgene, chlorine, sulfur mustard, etc., were weaponized in Polat et al. (2018).

Arsenic (As) occurs in a variety of different chemical forms, among them volatile (gaseous) species, usually referred to as arsine and methylarsines. Arsine, the most toxic form of arsenic, exhibits some characteristics that may make it useful as a CW agent. At concentrations above 0.5 ppm, a garlic-like odor may be noted, but arsine is toxic at much lower concentrations. Acute arsine poisoning due to inhalation of arsine gas (AsH_3) is rare but it has no known antidote. It is the most acutely toxic form of arsenic, causing rapid and severe hemolysis immediately on exposure. The mechanisms of hemolysis are not

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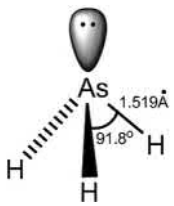
completely understood. Arsine has a short half-life (27–96 h) and is converted to various arsenic derivatives. A number of other arsine-derived organo-arsenic compounds have been developed and used as CW agents, including lewisite (L), methyldichloroarsine (MD), diphenylchloroarsine (DA), and ethyldichloroarsine (ED). It is also well known as an insecticide in the form of lead arsenate, arsenic acid, etc. and in pharmacy, especially in the form of salvarsan and neosalvarsan. Arsenic derivatives are also of value from the point of view of CW agents. However, on calculating the amount of arsenide required to establish a lethal concentration of arsine, this suggest no possibility of using the material in the field. Organic arsenic derivatives are the most important compounds from a military point of view. The first substance used was diphenylchloroarsine, a white solid, which readily penetrated the canister and caused sneezing. This was used alone, and in solution in diphenylchloroarsine. Later methyl and ethyl dichloroarsines were introduced (Beckett, 2008).

The arsines family includes compounds with various toxicities; however, arsenic trihydride or arsine is the most toxic form. It is a powerful hemolytic gas but has never been used as a chemical weapon because it exerts nonimmediate and nonpersistent toxicity. However, cases of occupational exposure are still reported despite strict regulation. This agent, whose mechanism of action is still not well defined, is poorly recognized because of the rarity of intoxication. Fast detection means are available, however health professionals still need to learn to recognize arsine intoxication in order to provide early, specific treatment and to avoid damage to health (Plantamura et al., 2011).

Arsenicals are considered a threat, not so much from large nation states but from smaller, less developed nations and/or by terrorist organizations. The relative ease of production coupled with their effectiveness against an unprotected population make organic arsenicals a continued threat in the 21st century. This chapter describes the human health aspects of arsine, some of the important organic arsenicals including inorganic arsenic and the current status of development of suitable therapeutic measures.

21.3 Arsine



Arsine (AsH_3) (arsenic hydride, arsenic trihydride, hydrogen arsenide, and arsenous hydride) is a colorless, extremely flammable gas with a garlic-like odor. The relative molecular mass of arsine is 77.95. Its boiling point is −62°C and vapor pressure at 20°C is 1043 kPa. Arsine is a strong reducing agent, deposits arsenic on exposure to light and moisture, and is easily transformed into other oxidized arsenic forms [e.g., As (III) and As (V)]. Arsine gas is colorless, odorless, and 2.5 times denser than air (Table 21.1). Arsine is a class of organoarsenic compounds of the formula $AsH_{3-x}R_x$, where R = aryl or alkyl.

It is used in the production of organic chemicals and lead storage batteries. Arsine is metabolized to trivalent arsenic as well as pentavalent arsenic. Arsenic (III) is methylated to monomethylarsonate (MMA) and dimethylarsinate (DMA). Arsine metabolites are mainly excreted via urine (Sattar et al., 2016).

Arsine, the most toxic form of arsenic, is a potential CW agent. It is believed that arsine causes massive hemolysis of red blood cells by (1) formation of hydrogen peroxide and adducts with oxyhemoglobin, (2) interaction with the sodium–potassium pump, red blood cell swelling and bursting, and (3) finally, massive hemolysis generally leads to renal failure (Sattar et al., 2016). Inhaled arsine gas is distributed rapidly and causes massive red blood cell hemolysis that can potentially lead to cellular hypoxia. Exposure to other arsenic compounds to which arsine is metabolized can induce lung, bladder, kidney, and skin cancer in humans. Occupational sources where exposures to arsine at levels sufficient to cause acute arsine intoxication have occurred include copper smelting and refinery bronzing process, chemical company cleaning of clogged drains, transistor industry, burnishing of metals, etc. Many processes including electrolyte refining, galvanizing, soldering, etching, lead plating, metal smelting, and extraction

TABLE 21.1 The physical and chemical properties of arsine.

Properties of arsine	
Molecular formula	AsH_3
Molar mass	77.95 g/mol
Appearance	Colorless gas
Density	4.93 g/L, gas; 1.640 g/mL (−64°C)
Melting point	−117°C (157K)
Boiling point	−62.5°C (210K)
Solubility in water	0.07 g/100 mL (25°C)
Molecular weight	77.95

may expose workers to toxic concentrations of arsine (Chein et al., 2006).

21.3.1 Synthesis of arsine

Arsine is formed whenever nascent hydrogen is released in the presence of arsenic or by action of water on a metallic arsenide. The formation of arsine can be described with the help of the following reaction (Radke et al., 2014).

$$3\,H_2 + HAsO_2 \rightarrow AsH_3 + 2\,H_2O$$

AsH_3 is also prepared by the reaction of As^{3+} sources with H^- equivalents (Bellama and Macdiarm, 1968)

$$4\,AsCl_3 + 3\,NaBH_4 \rightarrow 4\,AsH_3 + 3\,NaCl + 3\,BCl_3$$

Alternatively, sources of As^{3-} react with protonic reagents to produce arsine:

$$Zn_3As_2 + 6\,H^+ \rightarrow 2\,AsH_3 + 3\,Zn^{2+}$$

21.3.2 Metabolism of arsine

21.3.2.1 In animals

The main route of arsine excretion is via urine after metabolism. Arsenic was excreted exponentially in intravenously arsenite-administered mice and, after 24 h, less than 10% of the dose remained. On the other hand, arsenic arising from inhalation exposure to 180 mg/m^3 arsine for 120 min was excreted more slowly and after 24 h about 45% of the arsenic remained in the exposed mice. The major metabolites determined in urine were As (III) and As (V), MMA and DMA.

21.3.2.2 In humans

Arsenic has been detected in the tissues, blood, and urine of workers in the petroleum industry poisoned with arsine. Arsenic was also found in the liver at a concentration of 11.8 mg/g, spleen at 7.9 mg/g, kidneys at 3.2 mg/g, brain at 0.6 mg/g, and in the urine at 0.6 mg/m in a fatal case of arsine poisoning (Apostoli et al., 1997). As (III) also gets oxidized to As (V) in urine of humans exposed to arsine 1–2 days following exposure. Trivalent arsenic is methylated to MMA and DMA (Flora, 2011).

21.3.3 Mechanism of toxicity

Arsine gas causes rapid destruction of red blood cells leading to hypoxia and renal failure on inhalation. It also causes massive hemolysis of red blood cells by (1) formation of hydrogen peroxide and adducts with oxyhemoglobin, (2) interaction with the sodium–potassium pump, red blood cell swelling and bursting, and (3) finally, massive hemolysis generally leads to renal failure. Oxyhemoglobin (oxyHb) has been recognized as a necessary component for overall mechanism of AsH_3-induced hemolysis, as conversion of oxyHb to carboxyHb prevents hemolysis in erythrocytes exposed to AsH_3 (Sattar et al., 2016; Flora, 2011).

Sulfhydryl groups of glutathione (GSH) prevent hemoglobin oxidation and are essential for the maintenance of intact erythrocyte structure and a 60% decrease in reduced GSH level in erythrocytes was noted post arsine exposure.

Arsine reacts with the sulfhydryl group of Na^+K^+-ATPase, causing impairment in the sodium–potassium pump which subsequently causes red cell swelling and hemolysis (Fig. 21.1) (Flora, 2011). Profound abnormalities in membrane ultrastructure and in red blood cell volume also have been recently reported, which were manifested by potassium leakage, sodium influx, and increases in hematocrit in arsine-exposed red cells (Susan et al., 2019).

21.3.4 Effects on humans

Toxicity of arsine to humans was first demonstrated in 1815 when a German chemist accidentally inhaled arsine vapor during an experiment.

21.3.4.1 Acute arsine poisoning

Persons exposed to arsine gas are often unaware because there are no symptoms at the time of exposure. The majority of cases of arsine exposure are clinically acute, however, lower levels of chronic exposure have been reported in the literature also (Casarett and Doull, 2001). Most patients report little or no discomfort at the time of exposure. Although a garlic-like odor may be noted with higher ambient arsine concentrations, serious toxicity may result

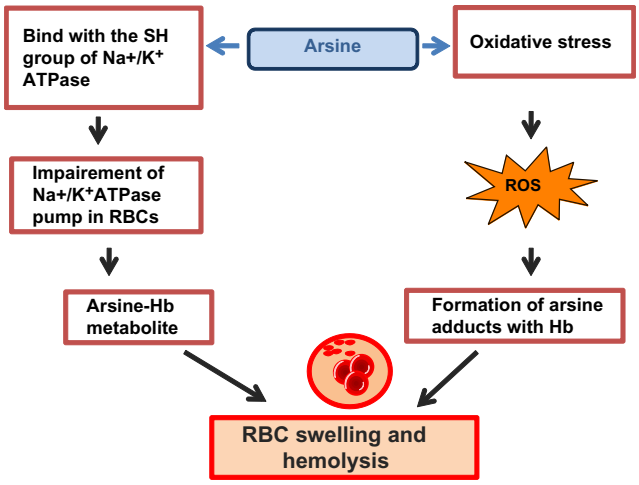


FIGURE 21.1 Hemolytic activity of arsine.

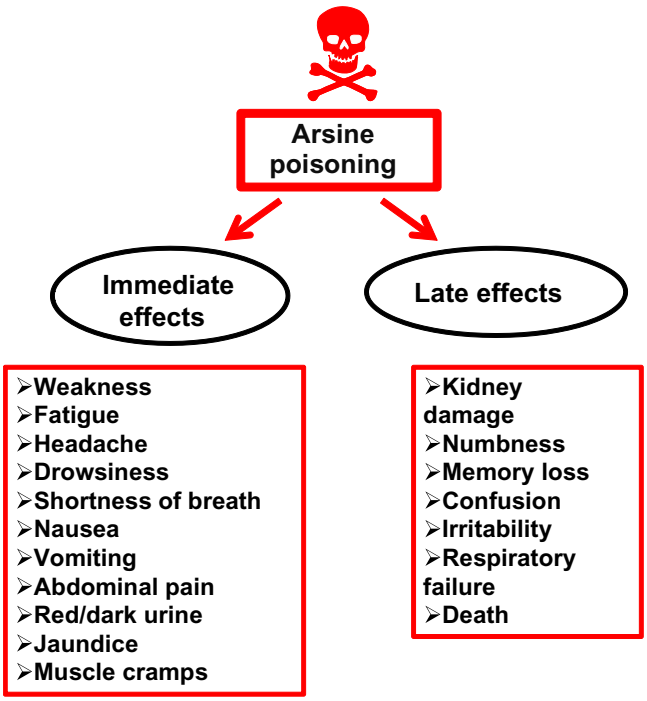


FIGURE 21.2 The signs and symptoms of arsine poisoning.

from clinically nondetectable exposures (Lenza, 2006; Song et al., 2006) (Fig. 21.2).

21.3.4.2 Immediate effects

Clinical manifestation of arsine intoxication appears within 24 h of exposure. Renal failure due to tubular destruction is an important consequence of arsine exposure. The urine is frequently discolored brown, red, or black, with hemoglobin in the urine thought to be the major cause. Urinalysis typically shows large amounts of protein and free hemoglobin with only a few red blood cells. The characteristic red/bronze tint of the skin is induced by hemolysis and may be caused by hemoglobin deposits. Hemolytic anemia is the most consistent clinical finding in humans. Massive hemoglobinuria may lead to anuria, which, if untreated, is often the cause of death.

21.3.4.3 Late effects

Late consequences of acute arsine poisoning include chronic renal damage, hematological changes, polyneuritis, and neuropsychological symptoms (e.g., irritation, confusion, memory losses, agitation, and disorientation). Peripheral neuropathy also has been reported within 6 months post-exposure (Sattar et al., 2016). An increase in total cell count and macrophages in bronchoalveolar lavage was observed in an arsine-exposed worker. Progressive improvement in diffusing capacity of lungs was observed only after 2 months of treatment.

21.3.4.4 Long-term exposure

Long-term exposure may cause symptoms similar to those observed in acutely poisoned individuals. The main differences include delay in onset and development of peripheral neuritis, development of gastrointestinal tract involvement, and the development of hemolysis and renal impairment (Sattar et al., 2016).

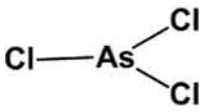
21.3.5 Diagnostic tests

Lab studies include complete blood count, methemoglobinemia, urinalysis, arsenic levels, electrocardiogram, and imaging studies.

Complications include hemolytic anemia, renal failure, hyperkalemia, and death.

Overwhelming exposures cause rapid death from massive hemolysis; most deaths occur from renal failure in patients who survive acute exposure.

21.4 Organic arsenicals



These arsenicals are a series of blister agents based around a chloroarsine ($AsCl_3$) molecule in which one of the chlorine atoms is replaced by an organic radical. These chloroarsines are effective cytochrome oxidase destroyers or blood agents. Arsenic seeks to replace calcium in the bones, thus causing bone marrow destruction as the endocrine system is concurrently attacked. Many organic radicals penetrate human skin, carrying their compounds with them (Cohen et al., 2006).

21.4.1 Mechanism of toxicity

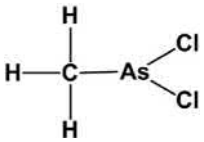
The exact mechanism for the toxic effects of organic arsenicals remains unknown. DNA alkylation and/or inhibition of glutathione-scavenging pathways are two postulated mechanisms (Nesnow et al., 2002). On contact with arsenicals a blistering reaction occurs on skin, eyes, or pulmonary tissues (Cohen et al., 2006). Animal data and limited human trials have suggested that organic arsenicals readily penetrate the skin. Within seconds of contact, the chemical fixes itself to the epidermis and dermis. The separation of dermis from epidermis together with capillary leakage causes fluid-filled vesicles (Naranmandura and Suzuki, 2008). Intravascular

hemolysis of erythrocytes with subsequent hemolytic anemia may result (Wu et al., 2003).

21.4.2 Symptoms

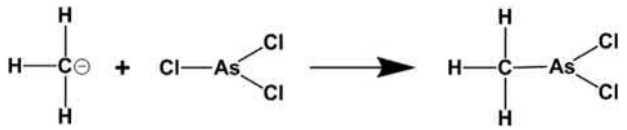
Vapor contact with the conjunctiva may be the victim's first symptom. Severe conjunctival irritation and blepharospasm may lead to loosening of corneal epithelial cells and swelling and edema of the cornea. Mucosal damage starts in the nose and descends down the respiratory mucosa in a dose-dependent fashion. Immediate pain, lacrimation, and irritation accompany the damage. DA vapor causes vomiting, which develops within 1−2 min after exposure to DA. The immediate onset of symptoms following exposure makes severe or systemic toxicity to organic arsenical unlikely. However, prolonged contact may lead to multi-organ involvement (Kinoshita et al., 2007).

21.5 Methyldichloroarsine



Germans referred to MD as methyldick, a toxic irritant, a vesicant, and a systemic poison. It causes pain, coughing, nasal discharge, frothing at the mouth, and irritation of the eyes, skin, nasal passages, and lungs. Inhalation of the vapor or dust produces a burning sensation along the entire respiratory tract and gasping for breath. Later, bloody expectorations are produced (Polat et al., 2018).

The structure of MD consists of a trichloroarsine ($AsCl_3$) molecule combined through catalyzation with a methyl (CH_3) group.



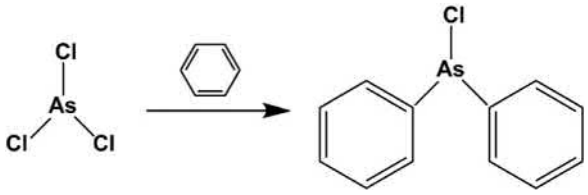
21.6 Dlphenylchloroarsine

DA is an irritating substance which was developed in 1918 for use in a smoke generator known as the M-device. DA has a very powerful irritant action on the mucous membranes of the eyes and nose, causes painful blistering of the skin, and is very dangerous for those working with it, since its vapor causes respiratory embarrassment, faintness, and long-lasting paralysis and anesthesia of the extremities (Kato et al., 2007).

As DA and related compounds cause intense effects on the nasal and upper respiratory passages, they are referred to as "sneeze gas" (sternutators).

21.6.1 Structure

DA is prepared from trichloroarsine ($AsCl_3$):

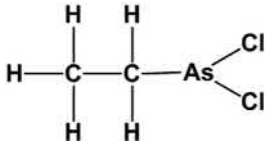


Two chlorine ions are replaced by benzene groups, forming a stable compound which may be safely stored under all field conditions.

21.6.2 Effects of dlphenylchloroarsine

The immediate effects of DA are those associated with tear-gas compounds: severe irritation to the eyes, nose, and throat. Severe headache and feelings of tightness of the chest and bowels occur within a minute of inhalation of this compound. The headache rapidly develops into a general nausea which results in vomiting within 3 min. In closed or confined spaces DA can produce fatalities through first causing unconsciousness and then asphyxiation (Ochi et al., 2004).

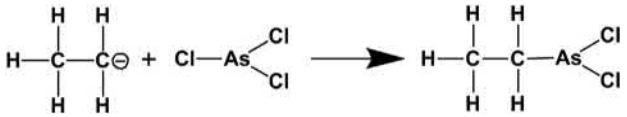
21.7 Ethyldichloroarsine



ED is fast acting compared to mustard or phosgene. ED is a colorless liquid that smells like rotting fruit and has multiple effects on the body. Within seconds of contact with the skin, the agent fixes itself to the epidermis and dermis, causing immediate pain. The agent penetrates deeper into the skin layers causing the destruction of subcutaneous tissue and fluid-filled blisters after prolonged exposure. Inhalation can cause pulmonary edema or "dry-land drowning." A lethal exposure, however, depends upon the period of exposure. A dose of 3000−5000 mg min/m^3 is generally lethal.

21.7.1 Structure

The structure of ED consists of a trichloroarsine ($AsCl_3$) molecule combined through catalyzation with an ethyl (C_2H_5) group.

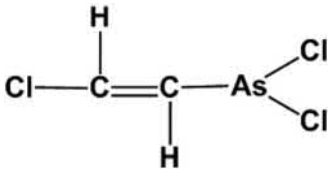


Production of ED is similar to that of MD, involving the ethylation of a chlorinated arsenite or arsenate salt, or in reductions of arsenious oxide, As_4O_6, a naturally occurring compound (Bartelt-Hunt et al., 2006).

21.7.2 Effects of ethyldichloroarsine

Chlorine bonds in ED result in its blistering, lachrymatory, and harsh respiratory effects. Dosages as low as 5 mg min/m^3 may cause severe discomfort to the eyes and throat. Sublethal dosages are detoxified by the body. ED's ethyl arsenic group may cause systemic damage to bone marrow and to the digestive and endocrine systems. Blisters may appear within 2–4 h following skin redness or rash. Like the mustards, ED actively attacks lung tissue and the damage is permanent to its survivors. ED causes permanent corneal damage.

21.8 Lewisite



Lewisite (chlorovinyldichloroarsine), a colorless, oily liquid at room temperature, has a faint "geranium-like" odor. It dissolves lowly in water and hydrolyzes rapidly to hydrochloric acid and lewisite oxide. Lewisite produces irritation and blistering of the skin and injury to the eyes and lungs after high exposure while, at lower levels, the effects resemble exposure to tear gas, with irritation of the skin, eyes, and respiratory tract. Chronic exposure may lead to development of chronic bronchitis and predispose to Bowen's squamous cell intraepithelial cancer of the skin (Sattar et al., 2016).

21.8.1 Background

Lewisite hydrolyzes in acidic medium forming hydrochloric acid (HCl) and chlorovinylarsenous oxide. Chlorovinylarsenous oxide is a compound which has a less potent blistering effect than lewisite. Trisodium arsenate, which is a toxic compound, may occur when lewisite is exposed to alkaline solutions. It is not used in industry. It is estimated that the LD_{50} (lethal dose, 50%) of lewisite is 30 mg/kg and the LC_{50} (lethal concentration, 50%) of lewisite is 100,000 mg min/mm^3 for dermatologic problems. Lewisite was discovered during WWI but has not been used in warfare because it hydrolyzes in water and the desired effect cannot be achieved in humid weather conditions. Lewisite is an agent which can penetrate ordinary clothes and rubber (Sattar et al., 2016).

21.8.2 Mechanism of action and toxicokinetics

Lewisite, a lipotropic agent, can be easily adsorbed via the skin and its systemic absorption may be lethal. Lewisite's major target organs are the skin, eyes, and airways. It causes a burning sensation, painful erythematous inflammation, and blisters on the skin. It leads to inhibition of pyruvate dehydrogenase by binding to the two thiol groups of lipoic acid. Pyruvate dehydrogenase is an enzyme which converts pyruvate to acetyl coenzyme A. Thus, lewisite causes a deterioration in oxidative phosphorylation. It also may lead to oxidative stress, DNA adducts, and apoptosis (Polat et al., 2018).

A no-observed-adverse-effect level (NOAEL) of 0.016 mg/kg/day in rabbits and 1.5 mg/kg/day in rats has been identified. Lewisite is capable of producing DNA damage; however, direct tests of its mutagenic potential have been inconclusive (Datta et al., 2007).

21.8.3 Clinical and pathological findings

Lewisite damages capillary walls; it causes hypovolemia and refractory hypotension. This phenomenon is called "lewisite shock" and causes death. Laboratory tests of the blood of persons exposed may show hemoconcentration; animal studies suggest elevated liver enzymes, including lactate dehydrogenase (Polat et al., 2018; Susan et al., 2019) (Fig. 21.3).

21.9 Inorganic arsenic

Arsenic is a metalloid belonging to group VA of the periodic table. It exists in three oxidation states: metalloid (0), trivalent (−3 or +3), and pentavalent (+5). The most common inorganic trivalent arsenic compounds are arsenic trioxide, sodium arsenite, and sodium trichloride. Pentavalent inorganic arsenic compounds are arsenic pentaoxide, arsenic acid, and arsenate, for example, lead arsenate and calcium arsenate. In general, the toxicity of arsenic compounds is in the following order: arsine > arsenites > arsenates > organic > elemental (Sattar et al., 2016).

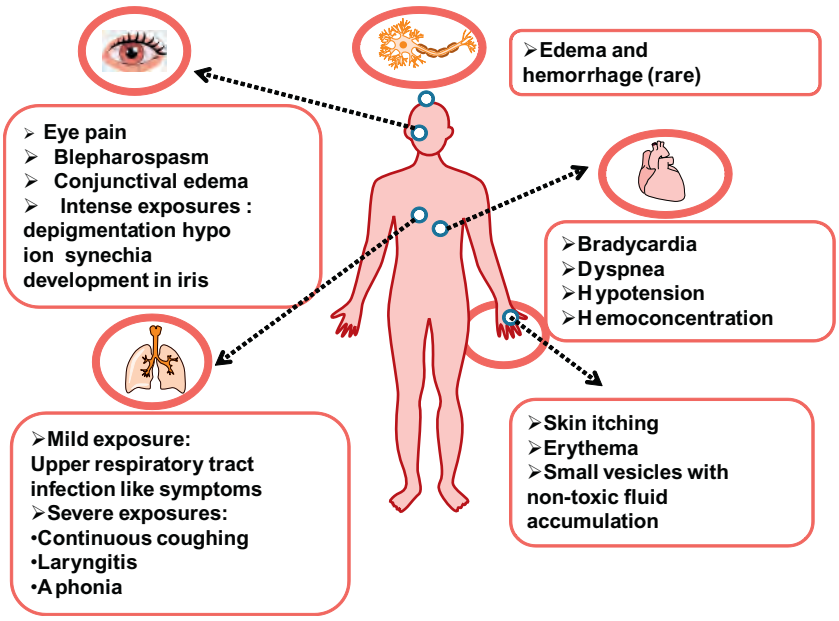


FIGURE 21.3 Lewisite toxicity in various organs.

21.9.1 Sources and uses

21.9.1.1 Uses

Arsenic is used as a bronzing and decolorizing agent in the manufacture of glass, and in the production of semiconductors (Tanaka, 2004), as a desiccant and defoliant in agriculture, and is a byproduct of the smelting of nonferrous metals, particularly gold and copper, from coal residues (Hall, 2002).

21.9.1.2 Exposure

Arsenic exposure occurs from inhalation, absorption through the skin, and primarily by ingestion of arsenic-contaminated drinking water. The exposure may come from natural sources, industrial processes (semiconductor manufacturing), commercial products, food, or medicines (Kosnett, 2013). Acute arsenic poisoning is limited to homicidal or suicidal attempts.

21.9.2 Toxicokinetics

Respiratory absorption of arsenic is a two-stage process, involving deposition of the particles on to airway and lung surfaces, followed by absorption of arsenic from deposited particulates. Trivalent and pentavalent inorganic arsenic have been reported to cross the placenta in laboratory animals and humans. The main form of arsenic bound to rat hemoglobin is dimethylarsinic acid (DMA), the primary metabolite of inorganic arsenic (Lu et al., 2004).

Exposure to either arsenite or arsenate leads to an initial accumulation in the liver, kidneys, and lungs. There have been many reports of arsine poisoning in workers. Findings indicate induction of acute kidney injury by arsine. The evidence suggests incidents of acute arsine poisoning are complicated by acute kidney injury (Lee et al., 2013). Clearance from these tissues is rapid, and long-term retention of arsenic is seen in hair, skin, squamous epithelium of the upper gastrointestinal tract, epididymis, thyroid, lens, and skeleton. Arsenic metabolism is characterized by two types of reaction: (1) reduction of the pentavalent arsenic to trivalent arsenic which is catalyzed by arsenate reductase (Radabaugh et al., 2002), and (2) oxidative methylation reactions in which trivalent forms of arsenic are sequentially methylated to form mono-, di-, and trimethylated products using *S*-adenosyl methionine as the methyl donor and GSH as an essential co-factor (Vahter, 2002). Pentavalent arsenic has been reported to be less toxic than inorganic trivalent arsenic. Metabolic methylation had historically been considered as a detoxification process, as shown in Fig. 21.4. Recently, it has been established that trimethylated arsenicals, particularly monomethylarsinous acid [MMA(III)] and dimethylarsinous acid [DMA(III)], which exist as intermediates in the metabolic methylation process of inorganic arsenic in humans (Fig. 21.4) (Mandal et al., 2001), are more active than the parent inorganic arsenic for enzymatic inhibition, cytotoxicity, and genotoxicity. The major route of excretion following exposure to inorganic arsenic is via the kidneys (Csanaky and Gregus, 2005).

21.9.3 Biochemical and toxic effects

21.9.3.1 Hematopoietic

Varieties of hematological abnormalities, basophilic stippling, increased bone marrow vascularity, and rouleau

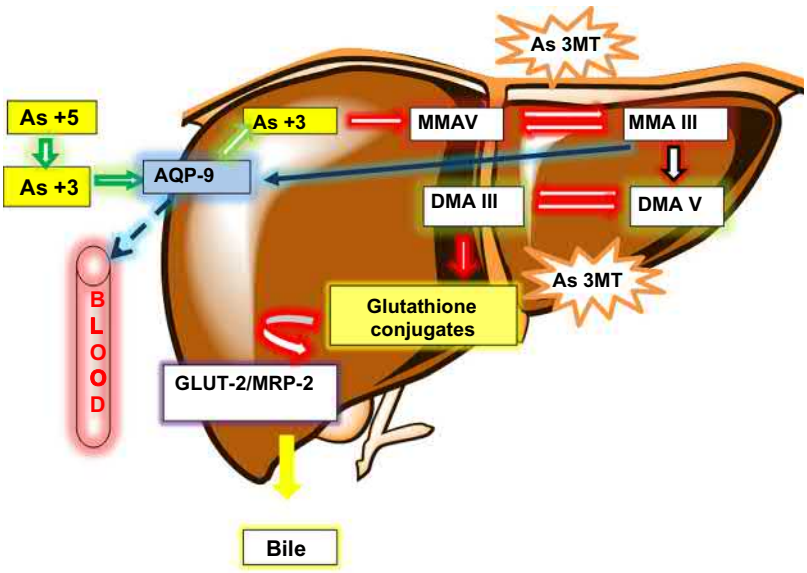


FIGURE 21.4 Detoxification process of arsenic in humans.

formation have been reported. These effects may be due to a direct hemolytic or cytotoxic effect on the blood cells and the suppression of erythropoiesis, and are mediated through depletion of intracellular GSH, resulting in the oxidation of hemoglobin. Subchronic exposure to arsenic affects the heme synthesis pathway (Flora et al., 2002) including inhibition of δ-aminolevulinic acid synthetase (ALA-S) and ferrochelatase activities, leading to increases in uroporphyrin (URO), coproporphyrin, and COPRO urinary excretion. Arsenic alters heme metabolism as shown by an inversion of the urinary COPRO/URO ratio. Profile of urinary porphyrins is recommended as early biomarkers for arsenic toxicity.

21.9.3.2 Skin (dermal)

Arsenic-induced dermal changes include hyperpigmentation, melanosis, hyperkeratosis, warts, and skin cancer (Rossman, 2003). Arsenic-exposed skin cancer occurs mostly in unexposed areas such as the trunk, palms, and soles. The most common skin cancers include Bowen disease, squamous cell carcinomas, basal cell carcinomas, and combined forms. Brittle nails, the surfaces of which are marked by transverse bands (leukonychia striata arsenicalis transverses), are characteristic bands of arsenic and are known as *Reynolds Aldrich-Mees lines*.

21.9.3.3 Hepatic

Arsenic is one of the first chemical agents to which liver disease was attributed in humans. Early symptoms in patients with arsenic-induced hepatic injury include bleeding esophageal varices, ascites, jaundice, or simply an enlarged tender liver. Changes in hepatic enzymes occur on arsenic exposure and these enzyme changes involved in the antioxidant defense system and membrane damage due to lipid per oxidation precede the pathomorphological lesions of arsenic-induced hepatic fibrosis in mice (Flora, 2011).

21.9.3.4 Gastrointestinal

Gastrointestinal symptoms are common during acute poisoning. Gastrointestinal effects due to chronic arsenic poisoning are called arsenicosis. High levels of exposure to arsenic dusts or fumes result in nausea, vomiting, and diarrhea. Patients complain of a metallic taste and garlic odor (Rosenberg et al., 2016). The toxic effects of arsenic on the gastrointestinal mucosal vasculature are vasodilatation, transduction of fluid into the bowel lumen, mucosal vesicle formation, and sloughing of tissue fragments. Rupture of vesicles may cause bleeding, profuse watery stools ("rice-water stools") and protein-losing enteropathy. The most likely mechanism of gastrointestinal toxicity is damage to the epithelial cells, with resulting irritation.

21.9.3.5 Respiratory

Respiratory disease is more common in patients with characteristic skin lesions due to chronic arsenic toxicity (Mazumder et al., 2000). Humans exposed to arsenic dust or fume inhalation often complaint of irritation of the mucous membranes, resulting in laryngitis, bronchitis, rhinitis, and trachea bronchitis, causing a stuffy nose, sore throat, dyspnea, chest pain, and chronic cough (ATSDR, 2000).

21.9.3.6 Cardiovascular

Chronic inhalation of arsenic trioxide may increase the risk of death in humans from cardiovascular disease.

Both acute and chronic arsenic exposure cause altered myocardial depolarization and cardiac arrhythmias that may lead to heart failure. Arsenic causes direct myocardial injury, cardiac arrhythmias, and cardiomyopathy. Two risk genotypes have been recently identified in humans: ApoE and Monocyte Chemotactic Protein-1 (MCP-1). When subjects were exposed to $>10\ \mu g/L$ the risk of carotid atherosclerosis was found to increase by greater than 10-fold (Flora, 2011). Excess intake of arsenic leads to a variety of vascular diseases such as blackfoot disease, Reynaud's phenomenon, cardio- and cerebrovascular diseases, and atherosclerosis (Sattar et al., 2016).

21.9.3.7 Reproductive and developmental

Arsenic readily crosses the placenta and may cause malformations, intrauterine death, and growth retardation. Spermatogenesis and/or sperm function may be impaired by organic arsenicals (Sarkar et al., 2003). Ahmad et al. (2001) observed pregnancy outcomes in women chronically exposed to arsenic through drinking water.

21.9.3.8 Neurological

Arsenic crosses the blood–brain barrier and may lead to alternations in whole rat brain biogenic amine levels in animals (Tseng, 2004). Usually, peripheral neuropathy, sensory neuropathy (Hafeman et al., 2005), and encephalopathy are the initial complaints associated with acute arsenic poisoning (Rodriguez et al., 2003). Other neurological symptoms arising due to arsenic are primarily those of a peripheral sensory neuritis, predominantly numbness, severe paresthesia of the distal portion of the extremities, diminished sense of touch, pain, heat, and cold, and symmetrically reduced muscle power.

21.9.3.9 Diabetes mellitus

Noninsulin-dependent (type II) diabetes is the prevalent form of diabetes mellitus found in populations chronically exposed to inorganic arsenic from the environment (Sattar et al., 2016; Flora, 2011). Type II diabetes is characterized by insulin resistance of internal organs and peripheral tissues that results in impaired glucose utilization, and consequently, in abnormally high blood glucose levels between and especially after meals. Insulin resistance and β-cell dysfunction can be induced by chronic arsenic exposure and these defects may be responsible for arsenic-induced diabetes mellitus.

21.9.4 Mechanisms of toxicity

Arsenic in its free form generates free radicals resulting in lipid peroxidation, depletion of antioxidant enzymes, and DNA damage, thereby establishing oxidative stress as the major mechanism of As-induced toxicity and carcinogenicity (Sattar et al., 2016; Flora, 2011). Arsenite inhibits pyruvate dehydrogenase (PDH) activity, perhaps by binding to the lipoic acid moiety. Inhibition of PDH leads to decreased production of ATP. Inhibition of PDH may also explain in part the depletion of carbohydrates observed in rats administered arsenite. Methylated trivalent arsenicals such as MMA^{III} are potent inhibitors of GSH reductase and thioredoxin reductase. The inhibition may be due to the interaction of trivalent arsenic with critical thiol groups in these molecules. The reduction of arsenate to arsenite occurs in vivo. Another potential mechanism is the replacement of phosphate with arsenate. Oxidative injury causing damage to DNA molecules and various cell components, such as polyunsaturated fatty acid residues of phospholipids, amino acids, peptides, and proteins has been reported as one of the foremost consequences of arsenic exposure as they are susceptible targets of metal-induced ROS attack (Flora, 2011).

21.9.4.1 Oxidative stress

Oxidative stress has now been established as one of the major mechanisms involved in arsenic-induced carcinogenesis. A number of recent reports have provided direct evidence of inorganic arsenic-induced free radical formation or production of oxidative stress (Flora, 2011). Reactive oxygen species (ROS) that damage DNA in vitro are generated from iron released from ferritin. The results suggest that some clastogenic effects of arsenic are mediated via free radicals and could increase the production of ROS, activation of transcription factors (e.g., AP-1, *c-fos*, and NF-κB) and oversecretion of proinflammatory and growth-promoting cytokines, resulting in increased cell proliferation and finally carcinogenesis (Sattar et al., 2016). Increased ornithine decarboxylase activity is often interpreted as a biomarker for cell proliferation. Arsenite appears to have an effect on the cell cycle, which may alter cell proliferation. Amplification of the gene, which codes for the enzyme dihydrofolate reductase, is enhanced by arsenic.

21.9.5 Diagnosis

21.9.5.1 Clinical features

Clinical features like skin lesions and neuropathy are crude and imprecise indicators of the severity of poisoning. White striae in the fingernails are also a useful clue to the diagnosis of arsenic toxicity, these white striae are also known as "Mee's line." Other symptoms include anemia, leucopenia or pancytopenia, gangrene of the feet (blackfoot disease), hyperpigmentation, hypopigmentation, and hyperkeratosis. Keratosis and pigmentation are the characteristic skin lesions associated with arsenic

toxicity. They are typically exhibited by diffuse thickening of the palms and soles, alone or in combination with nodules, and the presence of raindrops in the form of numerous rounded hyperpigmented macules in the body (Mendez et al., 2016; Hunt, 2014). In severe cases, cracks and fissures may be seen in the soles (Ahsan et al., 2009). Leucomelanosis is another common skin lesion, consisting of hypopigmented macules with a spotty white appearance. They are preferable samples to detect and quantify exposure to arsenic, as absorbed arsenic accumulates in both hair and nails, and elevated levels are noted within a few weeks of acute poisoning. The arsenic content of the fingernails and toenails has also been used as a bio-indicator of past arsenic exposure, and fingernail arsenic has been reported to be significantly correlated with hair arsenic content. The segmental growth of the hair shaft also provides valuable information about the duration and type of arsenic poisoning.

Blood arsenic levels are highly variable. Blood arsenic, normally less than 1 μg/dL may be elevated on acute intoxication. This is probably the most important diagnostic test for detecting arsenic exposure. Arsenic metabolites (inorganic arsenic + MMA + DMA) in urine have also been used as biomarkers of recent arsenic exposure. Arsenic is cleared from blood within a few hours after being absorbed. A study showed that acute arsenic poisoning can also be confirmed by speciation analysis of arsenic compounds in the plasma and urine by HPLC-ICP-MS. Multiple blood purification methods can also be used as a combined treatment for acute arsine poisoning (Wu et al., 2010).

Clouded urine is frequently seen in the most severely arsenic-intoxicated patients as absorbed arsenic if primarily excreted through the urine. Increased urinary protein content and aberrant excretion of trace elements also signify enhanced renal dysfunction. Though estimation of urinary arsenic is a consistent biomarker of exposure, it possesses some drawbacks which include accurate sample collection time, the volume of urine to be voided, etc.

21.9.5.2 Other biomarkers

Laboratory tests should include complete blood count, liver and renal functional tests, and blood, nail, and urine arsenic levels. Table 21.2 illustrates various biomarkers for effective diagnosis of arsenic intoxication. Other

TABLE 21.2 Routes of exposure and relevant biomarkers for various arsenic compounds.

#	Compounds of arsenic	Route of exposure	Symptoms/biomarkers	References
Arsine				
		Inhalation	Vertical white lines on nails, discolored brown, red, or black urine, free hemoglobin in urine, red/bronze tint in skin	Pinto et al. (1976), James and Wood (2006)
Organic arsenicals				
1.	Methyldichloroarsine	Dermal exposure	Blistering in skin, "dry-land drowning"	Bennett and Dill (1994), Pitten et al. (1999)
2.	Dlphenylchloroarsine	Inhalation	Irritation in the mucous membranes of eyes and nose, painful blistering in skin	Ochi et al. (2004), Kato et al. (2007)
3.	Ethyldichloroarsine	Dermal, Inhalation	Fluid-filled blisters form in skin, pulmonary edema or "dry-land drowning"	Bartelt-Hunt et al. (2006), Henriksson et al. (1996)
4.	Lewisite	Dermal, Inhalation,	Irritation and blistering of the skin, immediate eye pain and blepharospasm	McManus and Huebner (2005)
Inorganic arsenic				
		Inhalation, dermal, Ingestion	Hyperpigmentation, melanosis, hyperkeratosis, warts, decrease in ferrochelatase, increase in hepatic 5-aminolevelinic acid synthetase activity, pulmonary edema, peripheral neuropathy, sensory neuropathy and encephalopathy, diabetes mellitus, clouded urine, sister chromatid exchange	Hafeman et al. (2005), Woods and Southern (1989), Rossman (2003), Mazumder et al. (2000), Hughes and Kitchin (2006), Ghosh et al. (2006)

biomarkers of arsenic exposure include nonerythrocyte porphyrin enzyme activities and urine transforming growth factor TNF-α, accompanied by induction of heme oxygenase, mitogen-activated protein kinases, the ubiquitin-dependent proteolytic pathway, and protein kinase C in various tissues. These tests are still being investigated in laboratories and their clinical usefulness remains to be proven (Flora, 2011).

Cytogenetic markers, such as chromosomal aberrations, contribute significantly toward detection of this carcinogen. People suffering from chronic arsenicosis have been shown to have increased chromosomal aberrations (Ghosh et al., 2006) and sister chromatic exchange. However, it is still not the most interesting biomarker to detect the early effects of arsenic and is difficult to use on a large scale in epidemiological studies given its cost and difficulty of implementation (Eslava, 2004). The presence of micronuclei (MN) in isolated bladder and buccal cells has been considered as possible target tissues from direct exposure to As from drinking water (Bonassi et al., 2007).

21.9.5.3 Treatment

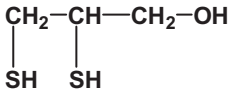
Highly specific treatment is required for poisoning with arsenicals, and there is no specific antidote for the treatment of arsine poisoning. Victims may be administered high-flow oxygen. One of the earlier strategies for the treatment of arsine poisoning involves stopping the ongoing hemolysis which may lead to renal dysfunction. Exchange transfusion is currently the treatment of choice. Chelation therapy generally is not recommended to reduce hemolysis, however chelating agents are shown to reduce arsenic in arsine-exposed subject. Since World War II, dimercapol has been the standard treatment for poisoning by arsenicals. DMPS (unithiol) and DMSA (succimer), dithiol water-soluble analogs of BAL, were mainly used for the chelation treatment of arsenic intoxication for more than half a century (Kosnett, 2013). However, treatment should be initiated as rapidly as possible because delay may result in a reduction of efficacy as the time interval between metal exposure and onset of chelation increases. DMPS and DMSA have a higher therapeutic index than BAL and do not redistribute arsenic or mercury to the brain. Although chelation may accelerate metal excretion, potential therapeutic efficacy in terms of decreased morbidity and mortality is largely not established in cases of chronic metal intoxication. We discuss below the efficacy of chelating agents for treating arsenicals, their drawbacks, and recent advancements in the area.

21.9.6 Chelating agents and chelation therapy

Chelators form a complex with the respective (toxic) ion and these complexes reveal a lower toxicity and are more easily eliminated from the body. This mechanism could be represented as: Metal − In vivo site + Chelating agent → In Vivo site + Metal-Chelating agent complex.

Chelation may thus be defined as the incorporation of a metal ion into a heterocyclic ring structure. Some of the chelating agents listed below have been reported to be useful in the treatment of arsenic.

21.9.6.1 2,3-Dimercaprol (dimercaprol; British antilewisite)



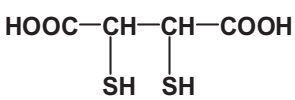
Dimercaprol or 2,3-dimercapto-1-propanaol, commonly referred to as BAL was originally developed to treat the effects of lewisite, namely systemic poisoning and local vesication. The empirical formula of BAL is $C_6H_8OS_2$ and its molecular weight is 124.21. It is an oily, clear, colorless liquid with a pungent, unpleasant smell typical of mercaptans and has a short half-life. The thiol groups present in BAL complex with arsenic and the resultant chelate is eliminated through the urine (Kosnett, 2013).

21.9.6.1.1 Drawbacks

BAL−arsenic complexes when oxidized result in the release of arsenic, which is detrimental to the system. In addition to rapid mobilization of arsenic from the body, it causes a significant increase in brain arsenic (Flora and Pachauri, 2010) and is also associated with nephrotoxicity, hypertension, tachycardia, and poor therapeutic index. Due to its oily nature, BAL requires deep intramuscular injection, which is extremely painful and causes an allergic reaction.

Two water-soluble analogs of dimercaprol have also been studied as lewisite antidotes. These are meso 2,3-dimercaptosuccinic acid (DMSA) and 2,3-dimercapto-1-propane sulfonic acid (DMPS). These two drugs circumvent two major disadvantages associated with treatment with BAL, that is, the need for intramuscular injection and limitation of dose by toxicity.

21.9.6.2 Meso 2,3-dimercaptosuccinic acid



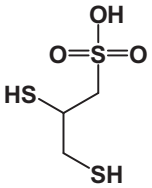
Another widely employed arsenic chelator is DMSA, which has low toxicity and does not redistribute arsenic to other organs. It chelates arsenic through two thiol groups. The empirical formula of DMSA is $C_4H_6O_4S_2$

and its molecular weight is 182.21. It is a weak acid and is soluble in water.

21.9.6.2.1 Drawbacks

Its use is limited by its inability to cross the cell membrane and hence it is not capable of chelating arsenic from intracellular compartments. Additional side effects such as gastrointestinal problems, placid neutropenia, elevated liver enzymes, and skin allergies have also been attributed to meso-DMSA administration (Susan et al., 2019).

21.9.6.3 Sodium 2,3-dimercaptopropane-1-sulfonate



DMPS, first introduced as "Únithiol" as a BAL analog, has the empirical formula $C_3H_7O_3S_3Na$ and molecular weight 210.3. It is a water-soluble derivative of BAL and has emerged as effective therapy against arsenic. It employs an organic anion transport pathway to permeate into the cell and form an insoluble complex with arsenic (Polat et al., 2018).

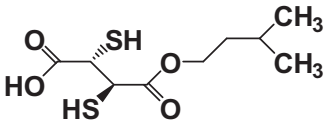
21.9.6.3.1 Drawbacks

Though no major adverse effects have been reported with the use of DMPS, mild effects such as headache, nausea, taste impairment, pruritis, and skin allergies have been reported. However, a dose-dependent decrease in copper content was found in the serum, liver, kidneys, and spleen (Susan et al., 2019). Oral administration of DMPS did not adversely affect late gestation, parturition, or lactation in mature mice or fetal and neonatal development.

21.9.6.4 Monoesters of meso 2,3-dimercaptosuccinic acid

Recently some mono- and diesters of DMSA, especially the higher analogs, have been developed and tested against experimental heavy metal poisoning to address the shortcomings of DMSA, particularly in depleting intracellular arsenic (Flora et al., 2007b,c; Flora, 2011). Derivatives of DMSA with better permeability across cell membranes have been developed and are currently in different phases of clinical and preclinical trials. These include monoisoamyl DMSA (MiDMSA), monocyclohexyl DMSA (MchDMSA), and monomethyl DMSA (MmDMSA) (Flora, 2011; Susan et al., 2019).

21.9.7 Monoisoamyl DMSA



MiADMSA is a lipophilic derivative that retains high affinity for arsenic as its parent compound. It chelates arsenic through the sulfhydryl groups and does not redistribute the same to other organs. Its lipophilic nature enables it to chelate both extracellular as well as intracellular arsenic. It has also been found to reverse arsenic-induced oxidative stress and neurotoxicity. Monoisoamyl DMSA (MiADMSA) is a new and one of the most effective of the vicinal class of metal-mobilizing agents (Flora, 2011). No reports are available for the therapeutic efficacy of MiADMSA against lewisite toxicity and other arsenicals. However, we reported the effect of MiADMSA on the reversal of gallium arsenide (GaAs)-induced changes in hepatic tissue (Flora et al., 2002). MiADMSA was found to be better than DMSA in mobilizing arsenic and in the turnover of the GaAs-sensitive biochemical variables. Plasma kinetics of MiADMSA (plasma-free drug and total drug) at 50 and 100 mg/kg p.o. was carried out. MiADMSA at a 50 mg/kg dose administered orally provided about 45% and 75% protection against oxidative stress and in lowering body arsenic burden. Also, pharmacokinetic analysis supported prolonged availability of the drug through oral administration. Collectively, these findings led to the conclusion that oral administration of MiADMSA was more effective than intraperitoneal administration and that the minimum effective dose with the least side effects was 50 mg/kg (Flora et al., 2012).

Arsenic-induced free radical generation in rat neuronal cells, leading to diminished mitochondrial potential and enzyme activities of all the complexes of the electron transport chain responded favorably to the treatment of MiADMSA (Dwivedi et al., 2011). MiADMSA significantly reversed the As-induced alterations in behavior and biochemical variables suggestive of oxidative injury. Arsenic-exposed rats showed significant differences in behavioral functions and water maze learning. Arsenic toxicity leads to various skin manifestations and arsenic accumulation in keratinized tissue. Pretreatment of HaCaT cells with MiADMSA elicited significant protection against arsenic-induced oxidative stress and apoptotic cell death in vitro. The findings are of clinical relevance and suggest MiADMSA to be a promising candidate in protecting skin against arsenic-induced toxic effects (Pachauri et al., 2013). Human embryonic stem cell-derived embryoid bodies were used to test the efficacy of MiADMSA against arsenic poisoning. It was concluded that the data generated using human ES cells are highly

comparable with the in vivo animal model. The efficacy of MiADMSA against arsenic is mainly attributed to the ability of formation of an adduct utilizing the sulfhydryl and carboxyl groups (Flora and Mehta, 2009).

The above results suggest that MiADMSA may be a future drug of choice owing to its lipophilic character and the absence of any metal redistribution. However, significant copper loss requires further studies (Mehta et al., 2006). Moderate toxicity after repeated administration of MiADMSA may be reversible after the withdrawal of the chelating agent.

21.9.7.1 Drawbacks

It is reported that the toxicity of DMSA, with an LD_{50} of 16 mmol/kg, is much lower than the toxicity of MiADMSA with an LD_{50} of 3 mmol/kg but lower than BAL (1.1 mmol/kg). However, mild side effects such as depletion of the essential metal ion pool including copper and zinc in the body and induction of mild anemia have been reported with MiDMSA and some biochemical alterations to the hepatic tissue in female as compared to male rats (Mehta et al., 2006).

21.9.7.2 Role of antioxidants

Oxidative stress can be partially implicated in arsenic toxicity and a therapeutic strategy to increase the antioxidant capacity of cells may fortify the long-term effective treatment of arsenic poisoning. This may be accomplished by either reducing the possibility of metal interacting with critical biomolecules and inducing oxidative damage, or by bolstering the cells' antioxidant defenses through endogenous supplementation of antioxidant molecules (Susan et al., 2019; Flora et al., 2013). Although many investigators have confirmed arsenic-induced oxidative stress, the usefulness of antioxidants along or in conjunction with chelation therapy has not been extensively investigated yet. N-acetylcysteine (NAC) is a thiol, a mucolytic agent, and a precursor of L-cysteine and reduced glutathione. NAC is a source of sulfhydryl-containing antioxidant that has been used to mitigate various oxidative stress conditions. Combined administration of NAC and succimer post arsenic exposure led to a significant recovery in biochemical variables indicative of oxidative stress and arsenic depletion from soft organs (Flora, 2011; Kannan and Flora, 2006). Various vitamins have been found to reduce the toxic manifestation of heavy metals (Flora, 2011; Flora et al., 2013). It was observed that vitamin E prevented the arsenite-induced killing of human fibroblasts. The protective mechanism of vitamin E could be attributed to its antioxidant property or its location in the cell membrane and its ability to stabilize membrane by interacting with an unsaturated fatty acid chain. The results suggested that vitamin C was better at providing clinical recoveries and vitamin E was equally efficient in decreasing the arsenic burden from tissues. An interesting study by Wei et al. (2005) reported the involvement of oxidative stress in DMA-induced bladder toxicity and proliferation in rat, and inhibitory effects of vitamin C on these alterations.

The influence of the coadministration of vitamin C or vitamin E was investigated upon the efficacy of two thiol chelators, DMSA or monoisoamyl DMSA, in combating chronic arsenic toxicity. Combined administration of vitamin C plus DMSA and vitamin E plus MiADMSA led to a more pronounced depletion of brain arsenic. Also, supplementation of vitamins led to a significant restoration of inhibited blood δ-aminolevulinic acid dehydratase (ALAD) activity and other oxidative stress parameters in liver, kidneys, and brain. These results suggested that coadministration of vitamin E or vitamin C may be useful in arsenic poisoning, although it has only a limited role in depleting arsenic burden. In another study, alpha-lipoic acid has been shown to have substantial antioxidant properties, when administered (70 mg/kg body weight) once daily for 60 days along with arsenic. The effect of alpha-lipoic acid on arsenic-induced oxidant production and lipid peroxidation level (LPO) in discrete brain regions of rats was also examined. The cortex, hippocampus, and striatum exhibited a greater increase of LPO levels than the cerebellum and hypothalamus. Also, simultaneous lipoic acid treatment along with arsenic proved to be sufficient in reducing oxidant production and the LPO level in all rat brain regions. In summary, the study demonstrated that arsenic-induced deficits in brain regions can be overcome through simultaneous treatment with lipoic acid (Shila et al., 2005). Garlic is another well-known folk remedy for a variety of ailments, however, very few studies are available suggesting its beneficial role against arsenic toxicity. In a study by Flora et al. (2009), the protective efficacy of aqueous garlic extract on parameters suggestive of hepatic injury, tissue oxidative stress, and mobilization of arsenic was investigated using two different doses. The results suggested that garlic extracts have a strong antioxidant property which could be beneficial in preventing arsenic-induced toxicity in cells. A study was conducted in rabbits to assess the oxidative injuries caused by arsenic toxicity and to evaluate the detoxifying effects of exogenous antioxidants, vitamins, zinc, selenium, or a plant polyphenol. The results indicated that arsenic induces toxicity in rabbits associated with an increase in lipid peroxidation and nitric oxide production in the body.

Administration of exogenous antioxidants such as polyphenols and recipe of vitamins, zinc, and selenium, however, was found to be useful for arsenic detoxification (Rabbani et al., 2003). Taurine (2-aminoethanesulfonic acid) has also been shown to exert a protective effect against arsenic-induced cytotoxicity in murine hepatocytes.

The cytoprotective activity of taurine against arsenic poisoning was found to be comparable to that of a known antioxidant, vitamin C. This further suggests that taurine protects mouse hepatocytes against arsenic-induced cytotoxicity (Sinha et al., 2007). Among various herbal extracts, *Moringa oleifera* (*M. oleifera*) seed powder also has been shown to restore arsenic-induced oxidative stress and reduce body arsenic burden. Hence it was concluded that concomitant administration of *M. oleifera* seed powder with arsenic could significantly protect against arsenic-induced toxic manifestations and thus could also be beneficial during chelation therapy with a thiol chelator (Susan et al., 2019). Concomitant oral supplementation of *Centella asiatica* (100, 200, or 300 mg/kg, orally once daily) during arsenic exposure (20 ppm in drinking water for 4 weeks) has also been shown to be an effective strategy against arsenic toxicity. More extensive studies are recommended for determining the effect of coadministration of *C. asiatica* during chelation therapy with a thiol chelator (Gupta and Flora, 2006).

Not only altered biochemical variables, but supplementation of antioxidants has also led to alleviation of arsenic-induced molecular alterations. Contamination of arsenic in drinking water is associated with several human diseases including cancer. In one study, a significant increase in the levels of protein oxidation, DNA strand breaks, and DNA−protein cross-links was observed in the blood, liver, and kidneys of rats exposed to arsenic (100 ppm in drinking water) for 30 days. However, upon coadministration of ascorbic acid and alpha-tocopherol to arsenic-exposed rats, a substantial reduction in the levels of arsenic-induced oxidative products of protein and DNA was seen. The results support the fact that free radical generation is one of the major mechanisms of arsenic-induced toxic manifestations and also suggest that ascorbic acid and alpha-tocopherol supplementation can improve the arsenic-induced molecular alterations (Kadirvel et al., 2007). Cardiac dysfunction has been shown to be associated with arsenic toxicity, which results in reduced cardiomyocyte viability, increased ROS production and intracellular calcium overload, and induced apoptotic cell death by mitochondrial dependent caspase-3 activation and poly-ADP ribose polymerase (PARP) cleavage. All these changes were found to be associated with increased IKK and NF-kappaB (p65) phosphorylation. Arsenic also markedly elevated the activity of p38 and JNK MAPKs. Taurine effectively suppressed these apoptotic actions, suggesting its protective role by attenuation of p38 and JNK MAPK signaling pathways. The results suggest that taurine effectively prevented arsenic-induced myocardial pathophysiology, attenuated NF-kappaB activation via IKK, p38, and JNK MAPK signaling pathways, and hence could be an effective therapy against As-induced cardiovascular burden (Flora, 2011; Susan et al., 2019).

21.10 Combination treatment

In an effort to improve the therapeutic index of the chelating agents, a combination of these agents has also been investigated. A combination of DMSA and MiDMSA was found to effectively abrogate the deleterious effects of arsenic exposure in guinea pigs. The combination was able to reverse oxidative stress-induced calcium influx in neurons either by deactivation of L-type calcium ion channels or scavenging calcium ions. Recently, MiDMSA was encapsulated in 50-nm polymeric nanoparticles and was found to be more efficient in mitigating the adverse effects of arsenic-induced toxicity in rodent models. Research has also revealed that zinc and selenium have the ability to increase the elimination of arsenic, thereby reducing the toxic manifestations of arsenic. Hence, concomitant administration of these ions with the chelating agents has also been attempted as a therapeutic stratagem (Susan et al., 2019).

As discussed above, metal chelators are given to increase the excretion of arsenic but, unfortunately, the uses of these chelators are comprised by a number of drawbacks. These drawbacks have opened the search for new treatments that have no side effects. A number of strategies have been discussed (Kalia and Flora, 2005). Among these strategies, combination therapy is a new and a better approach to treat cases of metal poisoning (Flora et al., 2007c; Mishra et al., 2008). In one study, we investigated whether coadministration of thiol chelators, like DMSA or sodium 2,3-dimercaptoprpane 1-sulfonate (DMPS), along with a newly developed thiol chelator, monoisoamyl DMSA, is more beneficial than monotherapy with these chelators, in counteracting chronic arsenic toxicity (Flora, 2011). It was concluded that concomitant administration of DMSA, a chelator known for its extracellular distribution with lipophilic chelators like MiADMSA, could play a significant and important role in abating a number of toxic effects of arsenic in animals compared to treatment with these chelators alone. We suggested that analogs with a long carbon chain (MiADMSA and MchDMSA) are better chelators than those with shorter carbon chains (MmDMSA) or DMSA. It is assumed that analogs of DMSA eliminate arsenic simultaneously from the cell and provide assistance in bringing GSH homeostasis toward normalcy. Further combinational therapy with DMSA and MiADMSA or MchDMSA proved more beneficial than combined treatment with MmDMSA and DMSA (Mishra et al., 2008).

Generation of ROS is one of the major mechanisms for arsenic-mediated oxidative stress; therefore, a study was conducted to explore the kinetic relationship of ROS with calcium and to dissect the calcium ion channels responsible for calcium imbalance after arsenic exposure. Another crucial aspect investigated was whether mono- or

combinational chelation therapy prevents arsenic-induced neuronal apoptosis in guinea pigs. The results indicated that arsenic caused a significant increase in ROS followed by NO and calcium influx, which was mainly dependent on L-type voltage-gated channels that disrupt mitochondrial membrane potential, and increase bax/bcl2 levels and caspase-3 activity leading to apoptosis. Another interesting and useful finding was that combinational therapy of DMSA and MiADMSA was most effective in reversing arsenic-induced alterations. The results provide strong evidence for the role of L-type calcium channels in regulating arsenic-induced calcium influx and that DMSA + MiADMSA combinational therapy may be a better and much more effective strategy than monotherapy in mitigating chronic arsenicosis (Pachauri et al., 2013).

As only few experimental evidences are available, there is a need for in-depth investigation in this area. It is thus proposed to investigate the effects of combination therapy, particularly in the case of chronic arsenic poisoning, where a strong chelating agent is administered along with another structurally different chelating agent (Kalia and Flora, 2005) to evaluate whether combination treatment is able to promote the elimination of arsenic and restore arsenic-induced biochemical and clinical alterations.

21.11 Concluding remarks and future directions

The use of arsenicals remains a potential threat as they are relatively easy to manufacture and may cause significant morbidity and mortality. Lewisite is one of the arsenicals that has not been studied in detail with very little information available on the detailed toxic effects of organic arsenicals, particularly carcinogenicity, mutagenicity, and teratogenicity. It is an organic arsenical war gas which is a vesicant that has been reported for its ability to bind with a thiol group leading to the possibility of its undesirable effects on a variety of enzymes. Thus there is a very strong possibility that exposure to lewisite might also lead to carcinogenic effects, however this hypothesis requires experimental and epidemiological evidence. It is not known whether lewisite is persistent. However, arsenic is an elemental poison and any residual hydrolysis, combustion, and decontamination product is likely to contain an arsenical compound. Some of the major thrust areas for future research directions include the possibility of a delayed or latent effect arising after organic arsenical (particularly lewisite) exposure. These effects, despite having been studied in detail recently after inorganic arsenic exposure, have very little information available after organic arsenical exposure. The development of antidotes is another area which requires immediate attention. Although one approved antidote, BAL, is available for lewisite, further investigations are required with other derivatives of BAL such as DMSA, DMPS, or monoesters of DMSA like MiADMSA. Chelators are generally not of any immediate benefit as far as toxicity of arsine and once hemolysis has begun. With some new chelating agents at experimental stages like MiADMSA, the effectiveness and safety results of these chelating agents against arsine poisoning is needed. The toxicodynamics of arsine is also an area which requires exploration as this information would be of immense help in developing suitable antidotes, particularly regarding hemolysis by removing or displacing arsine (Fig. 21.5).

Inorganic arsenic, particularly arsenic (III), is a well-documented potent carcinogen causing cancer of the bladder, lung, skin, and, possibly, liver and kidney. Because of failures in the attempts to study the carcinogenic effects of

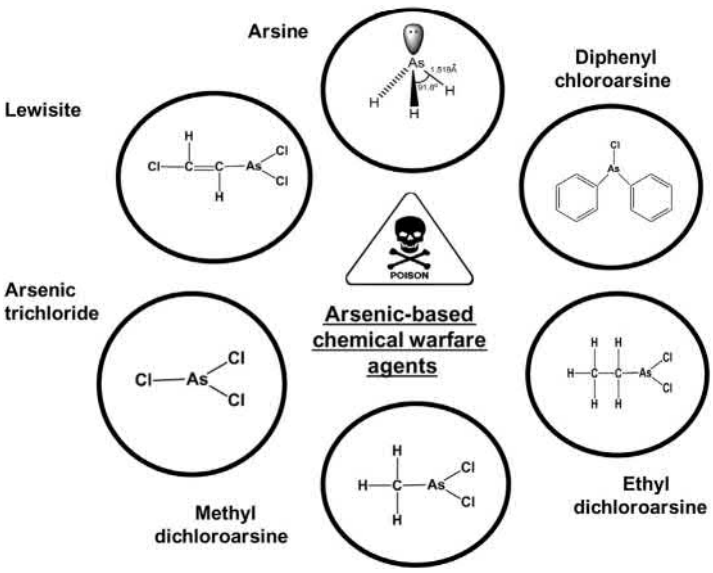


FIGURE 21.5 Arsenic-based chemical warfare agents.

arsenic in animal models, the mechanism of arsenic-induced carcinogenic effects remains unclear. The newly discovered potency of trivalent methylated arsenic metabolites opens up new opportunities for mechanistic studies. No treatment of proven benefit is currently available to treat chronic exposure. Treatment options advocated are vitamins, mineral supplements, and antioxidant therapy. The benefits of these treatment measures need to be evidence-based to receive endorsement and wider application. Further research work is also recommended in the areas of (1) the molecular mechanism of action of clinically important chelators, (2) intracellular and extracellular chelation in relation to the mobilization of aged arsenic deposits and the possible redistribution of arsenic to sensitive organs such as the brain, (3) the effect of metal chelators on biokinetics during continued exposure to arsenical, (4) combined chelation with lipophilic and hydrophilic chelators, (5) the use of antioxidants, micronutrients, or vitamins as complimentary agents or antagonists, (6) minimization of the mobilization of essential trace elements during long-term chelation, and (7) the fetotoxic and teratogenic effects of chelators.

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Chapter 22

Chlorine

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22.1 Introduction

Chlorine is a contact irritant in both humans and animals, and the respiratory tract is the primary initial target organ, with higher exposures affecting multiple internal organs. Very little chlorine gas exists in the environment due to its high reactivity and degradation by sunlight (Hov, 1985). Chlorine and sodium hydroxide are coproduced by the electrolysis of brines (the chlor-alkali process) using mercury, membrane, or diaphragm cells. The ion exchange membrane cell has become the predominant process, for economic and environmental reasons, and ~81% of global chlor-alkali capacity used membrane cell technology in 2017 (Burridge, 2005; CEH, 2018). Worldwide, there are over 500 chlor-alkali producers at over 650 sites, with an annual chlorine production capacity of 58 million tons (World Chlorine Council, 2012). Chlorine is one of the top 10 production volume chemicals in the United States, with over 10 million tons produced in 2010 and nearly that amount also produced in Europe in 2017 (U.S. Census Bureau, 2011; Euro Chlor, 2018).

Chlorine gas (Cl_2) has a pungent, suffocating odor and forms a greenish-yellow cloud near the ground when released in the environment due to its high vapor density (Rumble, 2018; O'Neil et al., 2013). The Cl_2 odor detection threshold varies considerably among humans. The mean value is approximately 0.2−0.4 ppm but values as low as 0.06 ppm have need detected, although odor perception tends to decrease over time (NIOSH, 1976; ATSDR, 2010). Chlorine gas is slightly water-soluble (~4.4 g/L; O'Neil et al., 2013), but it reacts quickly with water to form hydrochloric acid (HCl) and hypochlorous acid (HOCl). Selected chemical and physical properties of Cl_2 are listed in Table 22.1.

22.2 History of use and human exposure

Despite its potential hazards, chlorine continues to be produced, transported, and used in great quantities worldwide due to its great utility industrially and as a water disinfectant (Jones et al., 2010). The major uses of Cl_2 are in the manufacture of polyvinyl chloride and other plastics, chlorinated solvents, and pharmaceuticals; as a bleaching agent and biocide in pulp and paper production; and in water purification and waste treatment systems (Evans, 2005; CEH, 2018). Chlorine has been used as a water disinfectant in the United States since the early 1900s, dramatically cutting the death rate from typhoid fever (Toren and Blanc, 1997; Evans, 2005).

Use of chlorine gas as a chemical warfare agent began during World War I. The most notable release occurred near Ypres, Belgium, on April 22, 1915, when over 150 tons of chlorine gas were released in 5 min, killing an estimated 800 soldiers and incapacitating 2500−3000 others (Joy, 1997). Its use as a warfare agent declined after World War I because chlorine gas has significant warning properties (i.e., color and odor), allowing relatively easy avoidance to exposure. Its common use and availability, however, have made it easy to obtain in sufficient quantities to cause mass casualties in recent geopolitical conflicts. Chlorine gas has been used by terrorists in the Iraq war, with bombs being rigged to chlorine tanker trucks or cylinders intended for use in water treatment or for other industrial purposes (Wetz et al., 2007). More recently, chlorine gas has been used against both civilian and military targets in the war in Syria, where it was deployed in car or truck bombs or fired in rockets (Johnston, 2017; Devi, 2018; OPCW, 2014).

Accidental exposures to chlorine gas have occurred during its use as a water and surface disinfectant, and during its production and transport (primarily by rail). One of the deadliest accidents occurred on January 2005 in Graniteville, South Carolina, where nine people died and nearly 600 required medical attention when a chlorine tank ruptured from a train collision (Van Sickle et al., 2009; Jones et al., 2010; Mackie et al., 2014). Sites of recent documented human exposures included a

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TABLE 22.1 Chemical and physical data for chlorine.

Parameter	Value	References
Molecular formula	Cl_2	Rumble (2018)
Molecular weight	70.90	Rumble (2018)
CAS registry number	7782-50-5	Rumble (2018)
Synonyms	Bertholite, molecular chlorine, chlorine mol, dichlorine	ATSDR (2010)
Physical state	Greenish-yellow gas	Rumble (2018)
Odor threshold	0.2−0.4 ppm (mean)	ATSDR (2010)
Conversion factors in air	1 ppm = 2.9 mg/m^3; 1 mg/m^3 = 0.344 ppm	ATSDR (2010)
Boiling point	−34.04°C	Rumble (2018)
Melting point	−101.5°C	Rumble (2018)
Vapor pressure	5830 mmHg at 25°C	ATSDR (2010)
Vapor density	2.48 at 20°C	O'Neil et al. (2013)
Liquid density	1.565 g/cm^3 at −34°C	Rumble (2018)
Flash point/flammability	Not flammable	O'Neil et al. (2013)
Water solubility	4.4 g/L at 25°C	O'Neil et al. (2013)

wastewater treatment plant (Bellenger and Frizzi, 2014); a metal recycling facility (Harvey et al., 2018); a factory handling chlorine (Kim et al., 2014); and public swimming pools (Vajner and Lung, 2013; Matos et al., 2017). Healthcare workers at Ebola treatment centers in Africa who were exposed to excessive chlorine gas from its use as a disinfectant developed respiratory, eye, and skin disease (Carpenter et al., 2016; Mehtar et al., 2016). A review of the literature from 2007 to 2017 by Tuong et al. (2019) described hundreds of cases of accidental chlorine exposure.

Brzozowska (2016) conducted computer simulations of the impact of a chlorine tanker truck collision that released 10 tons of chlorine into the atmosphere on a bypass in the city of Bielsko-Biała, Poland. The simulations indicated that depending on the wind direction, there could be over 22,000 people injured and up to 5000 fatalities. Fatemi et al. (2017) assessed the impacts on surrounding residents of a simulated nighttime chemical release of 1 ton of chlorine over a 1-h period from a warehouse in Ray, Iran. The number of affected people by chlorine release in the spring, summer, autumn, and winter was estimated as 22,500, 25,000, 28,100, and 27,500, corresponding to chlorine dispersal distances of 8.3, 8.8, 7.6, and 6.4 km, respectively. To assist in validating models for decision response and risk mitigation, Sohn et al. (2019) conducted a series of experimental releases of pressurized chlorine gas to examine indoor−outdoor exchange, transport through a multiroom structure, and the first-order loss rate due to reaction or sorption.

22.3 Absorption, distribution, metabolism, and excretion

The vast majority of inhaled chlorine (>90%) is absorbed via the respiratory tract in humans and animals (Nodelman and Ultman, 1999a,b; Morris et al., 2005; Abdel-Rahman et al., 1983). Human studies showed that >95% of an inhaled bolus of 0.5−3 ppm chlorine was absorbed in the upper airway and <5% in the lower airway, regardless of the mode of breathing or respiratory flow rate (Nodelman and Ultman, 1999a,b). Absorbed chlorine is not subject to metabolic biotransformation, and it joins the endogenous pool of chloride ions distributed largely in the extracellular pool throughout the body. Using an in vivo neutron activation analysis system, Mohseni et al. (2016) determined that the short-term kinetic behavior of chlorine (^{36}Cl) in the human hand followed an exponential function corresponding to the rapidly exchangeable pool, with a mean redistribution half-life of 24.2 ± 8.5 min. Rats that were orally administered H^{36}Cl in distilled water excreted the majority of ^{36}Cl in the urine (36.43%) and feces (14.80%) over a 96-h postexposure period (Abdel-Rahman et al., 1983).

22.4 Mechanistic studies

In both humans and animals, the respiratory tract is the primary initial target organ for inhaled chlorine, which exists predominantly as HOCl and the hypochlorite ion (OCl^-) in the fluid lining the respiratory tract surface (O'Neil et al., 2013; ATSDR, 2010; Squadrito et al., 2010). It is believed that chlorine toxicity is due to its oxidant properties, and is mediated largely by the hypochlorite ion (Morris et al., 2005; ATSDR, 2010). An alternative model has proposed that Cl_2 itself can react with biomolecules in the epithelial lining fluid in the respiratory tract, and may be able to penetrate the lung surface (Squadrito et al., 2010). This hypothesis was based on a kinetic analysis that found that the reaction rate of chlorine with water was several orders of magnitude lower than its reaction rate with low-molecular-weight antioxidants and N-terminal functions of peptides and proteins in lung epithelial fluid (Squadrito et al., 2010).

Inhaled chlorine reacts with the epithelial lining of the airways and lungs. Depending on the chlorine exposure concentration and duration, respiratory toxicity can be manifested as sensory irritation, increased airway resistance, and respiratory tract lesions (Withers and Lees, 1985; Bitron and Aharonson, 1978). In rats and mice, chlorine sensory irritation decreases the respiratory rate by stimulating the trigeminal nerve endings in the respiratory mucosa (Alarie, 1981). A mouse model for the mechanism by which chlorine exposure causes reactive airways dysfunction syndrome (RADS) was developed by Jonasson et al. (2013a). Numerous rodent studies showed that acute chlorine exposure can cause lung fibrosis, leading to long-term impairment of lung function (Yildirim et al., 2004; Musah et al., 2012, 2019; O'Koren et al., 2013; Mo et al., 2013; Jonasson et al., 2013b; Wigenstam et al., 2016).

In addition to causing epithelial injury, the presence of HCl and HOCl in the airways causes an inflammatory response. The recruited neutrophils and macrophages increase the local concentration of nitric oxide (NO) and H_2O_2, which can react with HOCl and HCl to produce hydroxyl radicals and reactive intermediates able to nitrate, chlorinate, and dimerize aromatic amino acids (Olin et al., 1999; Evans, 2005; Jones et al., 2010). Chemically induced neutrophil depletion, but not eosinophil or macrophage depletion, abolished airway hyperresponsiveness (AHR) in large airways and prevented increases in antioxidant gene expression and NRF2 nuclear translocation in mice (McGovern et al., 2015). Ano et al. (2017) showed that Nrf2-dependent phase II enzymes (NQO-1 and GPX2) are involved in the resolution of airway inflammation and AHR after acute exposure to Cl_2 (5 min at 100 ppm). Glutathione deficiency exacerbated the acute inflammation but not AHR. Upregulation of UPR signaling (protein kinase RNA-like endoplasmic reticulum kinase, inositol-requiring enzyme 1a, activating transcription factor 6a) and expression of hepcidin was seen in the lungs and skin of mice within 6 h of exposure (Li et al., 2013). Chlorine-exposed mice had lung hyaluronan damage, manifested as an increase in low-molecular-weight fragments (L-HA, $<$300 kDa) in bronchoalveolar lavage (BAL) fluid, which was proposed to cause oxidative lung injury leading to AHR (Lazrak et al., 2015). Acute chlorine exposure caused impaired lung surfactant function, neutrophilia, altered BAL phospholipid content, and increased pulmonary elastance in mice (Massa et al., 2014). Coagulation abnormalities were proposed to play a role in chlorine-induced lung damage, as treatment with heparin reversed increases in protein levels and inflammatory cells in the BAL of mice (Zarogiannis et al., 2014).

The heart was identified as a target organ following chlorine exposure in humans and animals, and in ex vivo myocyte cultures (Carlisle et al., 2016). A follow-up study of people living in the area affected by the Graniteville, South Carolina, chlorine spill found an increase in hypertension-related hospital discharge rates (Howell et al., 2019). Acute exposure studies with rats (400−600 ppm for 27−30 min) showed cardiotoxic effects including increased lactate in the coronary sinus, decreased systolic and diastolic blood pressure, left ventricular systolic and diastolic dysfunction, and biventricular failure (Zaky et al., 2015); decreased cardiac output (Okponyia et al., 2018); and bradycardia accompanied by increased blood hemoglobin, red blood cells, hematocrit, and fibrin (Luo et al., 2014). Primary cardiomyocyte cultures showed decreased total ATP content and loss of sarcoendoplasmic reticulum calcium ATPase (SERCA) activity due to chlorination of tyrosine residues and oxidation of cysteine-674 (Ahmad et al., 2015). Cell death and inhibition of SERCA were decreased by added thiocyanate, genetic SERCA2 overexpression, and pretreatment with SERCA activators ranolazine and istaroxime.

Biomarkers of Cl_2 inhalation exposure were identified in studies with rats and mice. These included 8-isoprostane in airways and blood (Elfsmark et al., 2018), chlorinated lipids 2-chloropalmitaldehyde, 2-chlorostearaldehyde, 2-chloropalmitic acid, and 2-chlorostearic acid in the lungs and plasma (Ford et al., 2016); and phosphatidylglycerol chlorohydrins in BAL (Hemstrom et al., 2016). The ratio of nitric oxide (eNO) to carbon dioxide (eCO_2) levels in exhaled breath was diagnostic of the degree and time-course of pulmonary injury in rats exposed to chlorine gas (Luo et al., 2014). Other biomarkers included hemoconcentration (increased hemoglobin, red blood cells, and hematocrit) and increased BAL protein levels.

In vitro mechanistic studies with bacteria and mammalian cells have led to various theories for the chlorine mechanism of action, including direct interaction of chlorine or hypochlorite with enzymes, other proteins, and nucleotide bases, and disruption or degradation of cell membrane structure and function (U.S. EPA, 1994). Chlorine was proposed to cause systemic endothelial dysfunction by inhibiting endothelial nitric oxide synthase (eNOS)-dependent signaling, based on its inhibition of eNOS-dependent vasodilation in isolated rat aortas (Honavar et al., 2011). Pulmonary arteries (PA) isolated from chlorine-exposed rats had increased superoxide formation and inhibition of NO-dependent vasodilation, whereas chlorine-treated rats had decreased PA pressure that was reversed by the iNOS inhibitor 1400W (Honavar et al., 2014a). This suggests that disruption of NO signaling that maintains PA tone plays a role in postexposure toxicity. Upregulation of autophagy mitigated mitochondrial damage in NCI-H441 cells exposed to chlorine gas, and was negated by pretreatment with the autophagy inhibitor methyladenine (Jurkuvenaite et al., 2015).

22.5 Toxicity

The predominant and most sensitive target of chlorine is the respiratory tract, although it can also damage the skin, eyes, and internal organs at sufficiently high exposures (Evans, 2005). Toxicity resulting from single or multiple exposures to chlorine gas has been evaluated in humans and laboratory animals, which showed many similarities in their responses.

22.5.1 Human studies

The human effects of chlorine inhalation have been described in single-exposure case reports and controlled studies with volunteers, in repeated-exposure studies with volunteers, and in occupational chronic exposure studies (Table 22.2). In a summary of various anecdotal case reports, Winder (2001) noted that exposures of up to 3 ppm caused mild irritation; 5−15 ppm caused moderate irritation; >15 ppm caused severe irritation with pulmonary involvement; and death occurred from exposure to ≥430 ppm after 30 min or less. A selective review of publications from 1966 through 2016 found that the most common clinical features in civilians exposed to chlorine gas were cough (29%), dyspnea (22%), sore throat (16%), eye irritation (12%), and excessive sputum (7%) (Govier and Coulson, 2018). Full recovery occurred in most cases, and deaths were rare (<1%). Chlorine injury of the distal airways, which are repaired less efficiently, can lead to persistent respiratory symptoms and lung-function decrements (Hoyle and Svedsen, 2016).

Controlled human studies generally found that exposure to 0.5−2 ppm Cl_2 for a period of a few minutes to 8 h caused slight to moderate irritation of the eyes, nose,

TABLE 22.2 Human chlorine inhalation studies.

Exposure time	Concentration (ppm)	Effects	References
Single-exposure human studies			
15 min	0.5	At 0 and 15 min postexposure, NAR was ↑ in rhinitics but not normal subjects; rhinitics had greater nasal irritation and congestion; no effect on pulmonary peak flow, rhinorrhea, or headache in either group	Shusterman et al. (1998)
15 min	1.0	At 0 and 15 min postexposure, NAR was ↑ in rhinitics but not normal subjects; effect ↑ with age; very slight nasal irritation and odor perception	Shusterman et al. (2003a)
15 min	1.0	At 0 and 15 min postexposure, NAR ↑ in rhinitics but not normal subjects; no effect on nasal lavage fluid levels of mast cell tryptase, or plasma neuropeptides, albumin, urea, lysozyme, or 7F10-mucin	Shusterman et al. (2003b, 2004)
15 min	1.01 ± 0.034	No subject smelled the chlorine or had respiratory symptoms, and there was no effect on the FEV1 or airway responsiveness. Exposure was with a newly developed closed-circuit apparatus	Ojanguren et al. (2018)

(*Continued*)

TABLE 22.2 (Continued)

Exposure time	Concentration (ppm)	Effects	References
1 h	0.4	No pulmonary function changes in those with AHR or asthma	D'Alessandro et al. (1996)
1 h	1.0	Modest changes in pulmonary function measurements, greater in hyperreactive subjects (± asthma), of which 2/7 had "respiratory symptoms"	
15 min–2 h (left room every 15 min)	0.5, 1.0	Barely perceptible irritation of eyes, nose, and/or throat	Joosting and Verberk (1974)
	2.0	Distinctly perceptible irritation of eyes, nose, and throat; some coughing	
	4.0	Nuisance level irritation of eyes, nose, and throat; some coughing	
4 h + exercise 15 min/h	0.5	Moderate or mild itching or burning of throat and nose; severity generally ↑ with exposure time	Anglen (1981)
	1.0	Moderate or mild irritation of throat, eyes, and nose; runny nose; severity ↑ over time; few had pharynx injection, ↑ mucous secretion, and inspiratory rales	
	2.0	Moderate or mild irritation of throat, eyes, and nose; runny nose; severity ↑ over time; few had pharynx injection or palpebral fissures, ↑ mucous secretion, inspiratory rales, altered pulmonary function	
8 h + exercise 15 min/h; had 30 or 60 min break after 4 h	0.5	Moderate or mild itching or burning of throat and nose; severity generally ↑ with exposure time	Anglen (1981)
	1.0	Moderate or mild itching or burning of throat, eyes, and nose; runny nose; urge to cough; severity ↑ with exposure time; few had pharynx injection or palpebral fissures, ↑ mucous secretion, and inspiratory rales after 4 and 8 h; after 8 h had slight changes in pulmonary function	
8 h + exercise 15 min/h	0.5	Slight changes in pulmonary function measurements after 4 and 8 h; changes were greater in an atopic subject	Rotman et al. (1983)
	1	Itchy eyes, runny nose, mild burning in throat, and slight changes in pulmonary function after 4 and 8 h; changes were sufficient in an atopic subject to terminate exposure after 4 h due to dyspnea and wheezing	
Multiple-exposure human studies			
6 h/d for 3 d	0.1, 0.3, and 0.5	At unspecified concentration(s) had eye, nose, and throat irritation, wheeze, nasal congestion and mucous production; pulmonary function normal	Schins et al. (2000)
10.9 ± 2.8 yr	0.146 ± 0.287	Male workers had fatigue, anxiety, dizziness, and white blood cell count; ↓ hematocrit. No effect on chest X-ray or pulmonary function	Patil et al. (1970)
>10 yr	0.298 ± 0.181	Workers had ↓CO lung diffusing capacity but no effects on pulmonary function measurements or ↑ in pulmonary emphysema	Capodaglio et al. (1970)

(*Continued*)

TABLE 22.2 (Continued)

Exposure time	Concentration (ppm)	Effects	References
13.9 ± 9.3 yr	< 0.05 (mean)	Male workers had no ↑ in respiratory symptoms or abnormalities in pulmonary function or chest radiographs. Possible coexposure to low levels of H_2S, methyl mercaptan, SO_2, and particulates	Chan-Yeung et al. (1980)
8.9 ± 8.6 yr	0.18 (mean)	Male workers had ↑ frequency of wheeze, chest tightness, chest illness, and decrements in pulmonary function. No ↑ in cough, phlegm, or dyspnea. Possible coexposure to low levels of SO_2, CH_3SH, and particulates	Enarson et al. (1984)
<10–25 yr	1.7 ± 0.12	Workers had sore or dry throat, shortness of breath, chest tightness or pain, headache, wheezing, chronic rhinitis and tonsillitis, olfactory deficiency, chest X-ray abnormalities, and impaired pulmonary function	Shi (1990)
6.76 ± 6.86 yr	0.27 ± 0.05	Exposed workers had increased prevalence of cough, phlegm, productive cough, and wheezing, and lower mean values of FEV1, FEV1/FVC ratio, and PEF than unexposed subjects	Neghab et al. (2016)

↑, increase(d); ↓, decrease(d); *AHR*, airway hyperresponsiveness; *d*, day(s); *FEV1*, forced expiratory volume during the first second of the test; *FVC*, forced vital capacity; *h*, hour(s); *NAR*, nasal airway resistance; *PEF*, peak expiratory flow; *yr*, year(s).

and throat, and small decrements in pulmonary function [such as the forced expiratory volume in 1 s (FEV_1) and airway resistance (R_{aw})] (Anglen, 1981; Rotman et al., 1983; D'Alessandro et al., 1996; Shusterman et al., 1998; Schins et al., 2000). The severity of irritation increased with exposure duration and concentration, and effects were greater in subjects with preexisting respiratory conditions (such as asthma and AHR) and in smokers. Ojanguren et al. (2018), however, found that subjects exposed to 1 ppm for 15 min did not detect its presence. A number of cases of RADS have been reported, and all were seen in individuals with preexisting respiratory conditions and current or former smokers (Evans, 2005). Withers and Lees (1985) applied probit analysis to human and animal lethality data to estimate that chlorine concentrations of 250 and 100 ppm were lethal to 50% of a regular and vulnerable population (LC_{50}), respectively, that was exposed for 30 min with an inhalation rate of 12 L/min. Based on chlorine inhalation lethality data for eight species (mouse, rat, guinea pig, rabbit, cat, dog, sheep, and goat), Sommerville et al. (2009) estimated 2-min exposure lethality values (LCT_{50}) of 13,500 and 9500 mg min/m^3 for the military and general population, respectively.

Few repeat-exposure human studies have been conducted with chlorine gas. In controlled studies, subjects exposed to 0.1, 0.3, or 0.5 ppm for 6 h/day for 3 days had no pulmonary function decrements, but did report eye and respiratory tract irritation (Schins et al., 2000). Workers exposed chronically to <0.1 ppm experienced no chlorine-related toxicity; exposure to ~0.2–0.3 ppm had little or no effect on pulmonary function but did result in fatigue, anxiety, wheezing, and chest tightness; and exposure to 1.7 ppm was associated with numerous respiratory symptoms (including sore throat, shortness of breath, chest tightness, wheezing, and rhinitis), chest X-ray abnormalities, and pulmonary function decrements (Patil et al., 1970; Capodaglio et al., 1970; Chan-Yeung et al., 1980; Enarson et al., 1984; Shi, 1990). The severity of effects increased with the number of working years and was greater in smokers. A recent retrospective cohort study found that occupational exposure to 0.27 ppm chlorine gas was associated with an increase in respiratory symptoms and statistically significant decrements in pulmonary function (Neghab et al., 2016).

22.5.2 Laboratory animal studies

The majority of the chlorine exposure animal studies were conducted using rats and mice, although studies were also conducted using monkeys, dogs, rabbits, guinea pigs, cats, sheep, and goats. Studies with rabbits, rats, guinea pigs, and mice are summarized in Table 22.3 (single exposure) and Table 22.4 (repeat exposure).

TABLE 22.3 Animal single-exposure chlorine inhalation studies.

Exposure time	Concentration (ppm)	Effect	References
Rabbits			
30 min	50	Temporary ↓ in lung compliance	Barrow and Smith (1975)
	100–250	Lung edema, hemorrhage, emphysema, inflammation, impaired function	
	500–1000	LC_{100}	
45 min	600	IM injection of nitrite (1 or 10 mg/kg) at 30 min after Cl_2 exposure caused up to 50% less protein and neutrophil accumulation in the airways and no mortality (~35% mortality over 18 h in controls)	Honavar et al. (2017)
Guinea pigs			
15–30 min	200	Severe lesions of upper respiratory tract mucous membranes, emphysema, inflammation, and exudate in bronchioles	Faure et al. (1970)
Rats			
2, 10 min	1500	Lung edema with bronchial epithelial sloughing and airway wall leukocyte infiltration; some epithelial regeneration after 72 h	Demnati et al. (1995)
10 min	579–2248	LC_{50} = 1931, 690, 448 ppm for 10, 30, 60 min; majority died in 1 wk; were restless, had eye and nasal irritation, dyspnea, and lesions in nose, larynx, trachea, and lung; some lesions were reversible	Zwart and Woutersen (1988)
30 min	547–645		
60 min	322–595		
15 min	200	After 24 h had AHR and ↑ neutrophils, eosinophils, cytokines IL-1β and IL-18 in BAL; edema in lung and heart; dexamethasone ↓ neutrophils but not AHR. After 14–90 d had signs of lung fibrosis (↑ pulmonary macrophages, TGF-β expression in BAL, airway collagen deposition)	Wigenstam et al. (2016)
15 min	200	The biomarker for oxidative stress 8-isoprostane was found in exhaled breath concentrate from airways at 24 h postexposure	Elfsmark et al. (2018)
15 min	1330	Lung eosinophilic accumulation, edema, and bleeding, which became interstitial fibrosis and thickening of alveolar septa after 45 d	Yildirim et al. (2004)
27–30 min	500–600	Respiratory failure, ↓ cardiac output, ataxia and hypotonia, seizures, death. Oxygen treatment ↑ survival to 6 h but also ↑ severity of acute respiratory failure, acidosis, and hypercapnia, and did not improve neuromuscular, cardiac, or respiratory effects	Okponyia et al. (2018)

(*Continued*)

TABLE 22.3 (Continued)

Exposure time	Concentration (ppm)	Effect	References
30 min	184, 400	Labored breathing, expiratory grunting; 1 h postexposure had ↓ arterial oxygen pressure and ↑ CO_2 levels, and respiratory acidosis. Had lung necrosis and epithelial sloughing, neutrophil accumulation, and mild alveolitis; BAL fluid had ↑ albumin, IgG, IgM, and phospholipid levels (400 ppm), and ↓ levels of ascorbate and reduced glutathione	Leustik et al. (2008)
30 min	400	Chlorinated lipids ↑ in the lungs and the plasma (2-chloropalmitic acid, 2-chlorostearic acid, free and esterified) (only parameters evaluated)	Ford et al. (2016)
30 min	400	Isolated pulmonary arteries (PA) had ↑ superoxide formation and inhibition of NO-dependent vasodilation. PA pressures in anesthetized rats were ↓ but restored by iNOS inhibitor 1400 W	Honavar et al. (2014a)
30 min	400	Transient hypoxemia, severe epithelial pathology in airways; increased BAL protein, neutrophils, epithelia; lung edema; effects lessened by sodium nitrite given IP at 2–6 h postexposure	Yadav et al. (2011)
30 min	413	Bradycardia and ↓ respiratory rate; ↑ exhaled (eNO/eCO_2) ratios, which were best toxicity predictors; ↑ hemoglobin, red blood cells, hematocrit, and fibrin in blood within 1 d postexposure	Luo et al. (2014)
30 min	500	Reduction in heart rate and oxygen saturation; increased chloramine and chlorotyrosine in blood plasma. Primary cardiomyocites had decreased total ATP content and loss of sarcoendoplasmic reticulum calcium ATPase activity	Ahmad et al. (2015)
30 min	500–600	At 500 ppm had ↑ lactate in the coronary sinus; after 20 h had ↓ systolic and diastolic blood pressure and left ventricular systolic and diastolic dysfunction, even with oxygen treatment. At 600 ppm had biventricular failure 2 h postexposure and death	Zaky et al. (2015)
60 min	213, 268, 338, 427	LC_{50} = 293 (260–329) ppm; had eye and nose irritation, and after 60 min had lacrimation, rhinorrhea, and gasping; survivors had lower weight gain. Liver tissue mottling was the most common finding at necropsy	MacEwen and Vernot (1972)
60 min	6, 30, 60	Nasal lesions (necrosis, inflammation, hyperplasia, degeneration), severity increasing with concentration and greatest in more anterior regions	Peay et al. (2010)

(*Continued*)

TABLE 22.3 (Continued)

Exposure time	Concentration (ppm)	Effect	References
6 h	0.1, 1.0, 5.0, 10	Nasal epithelial inflammation and hyperplasia, olfactory necrosis and degeneration; severity increased with concentration and was greatest in anterior regions; lesions seen at ≥1.0 ppm persisted at least 5 d	Peay et al. (2010), Jarabek et al. (2010)
6 h	9.1 ± 1.02	Nasal cavity lesions (epithelial degeneration, ulceration, and necrosis); most severe in olfactory mucosa	Jiang et al. (1983)
16 h	63	All survived 5 mo postexposure; had gross changes in brain, lungs, heart, stomach, intestines, liver, kidneys, and lung adhesions	Weedon et al. (1940)
	250, 1000	Lacrimation, dyspnea; all died by 16 h at 250 ppm and 1.7 h at 1000 ppm; had lesions in brain, lungs, heart, liver, stomach, intestine, and kidneys	
24 h	0.25, 1.25, 2.5	Nasal necrosis/degeneration followed by inflammation and hyperplasia; severity increased with concentration and greatest in anterior regions	Peay et al. (2010)
Mice			
5 min	100	Had ↑ airway resistance (methacholine challenge), ↑ BAL neutrophils, macrophages, and protein; ↑ 4-hydroxynonenal in whole lung tissue; attenuated by AEOL-10150 (metalloporphyrin antioxidant)	McGovern et al. (2011)
5 min	100	Cl-induced neutrophilia; ↑ expression of NRF2 mRNA, superoxide dismutase-1, and heme-oxygenase 1. Neutrophil depletion abolished AHR in large airways, ↑ in antioxidant gene expression, and NRF2 nuclear translocation, but not ↑ L-17 in BAL. Depletion of eosinophils and macrophages did not prevent AHR	McGovern et al. (2015)
5 min	100	AHR, increased cells, and glutathione in BAL, dimethylurea given IP 1 h before/after exposure decreased BAL protein and glutathione levels and prevented lung lipid peroxidation	McGovern et al. (2010)
	200, 400	As at 100 ppm but more severe; had microscopic pathological changes in airway epithelium (BAL fluid and effect of dimethylurea not evaluated)	
5 min	100, 200, 400, 800	Dose-related increases in airway responsiveness, airway epithelial loss, alveolar damage and inflammation, leukocytes and nitrate in BAL fluid, nitric oxide synthase expression, and oxidation of lung proteins	Martin et al. (2003)

(*Continued*)

TABLE 22.3 (Continued)

Exposure time	Concentration (ppm)	Effect	References
7.5 min	800	After 6 h: mortality was 8/8; 6/41; 0/40, 0/40; had increased lung weights (pulmonary edema), BAL protein and IgM levels, neutrophil infiltration, and airway epithelial denudation—more severe for the shorter exposures. After 24 h: only lung weight returned to sham control levels	Hoyle et al. (2010)
15 min	400		
30 min	200		
60 min	100		
10 min	0.7–38.4	RD_{50} = 9.3 ppm; respiratory rate ↓ was dose-related and was attained at each concentration within 5–7 min	Barrow et al. (1977)
10 min	1.0–760	RD_{50} = 25 ppm; appearance of animals was not addressed	Barrow and Steinhagen (1982)
10 min	~2.5–1000	RD_{50} = 10.9 ppm; animal appearance was not addressed	Chang and Barrow (1984)
10 min	579–1654	LC_{50} = 1034 and 517 ppm for 10 and 30 min, respectively. Animals had signs of restlessness, eye and nasal irritation, dyspnea, and ↑ lung weight	Zwart and Woutersen (1988)
30 min	458–645		
15 min	0.8, 2.0, 3.1, 3.8	RD_{50} = 2.3 ppm; dose-related ↓ in respiratory rate (30%–80% of baseline) and ↑ in airway resistance (64%–186%) and length of the expiratory pause (20–522 ms)	Morris et al. (2005)
15 min	25, 50, 100, 200	Concentration-related inflammatory response within 24 h postexposure with ↑ expression of IL-1β, IL-6, and CXCL1/KC in BAL fluid, ↑ in lung permeability, neutrophilic inflammation, fibrinogen, PAI-1, and cytokine 8-isoprostane, biomarker for oxidative stress	Elfsmark et al. (2018)
15 min	50, 200	After 6–12 h had lung edema, central and peripheral AHR, inflammation with macrophages and neutrophils. Inflammatory cells were found for ≥7 d and AHR for ≥28 d	Jonasson et al. (2013a)
15 min	200	Anorexia, lethargy, hypothermia; acute lung edema, collagen deposition (fibrosis), and inflammation; dexamethasone (IP) reduced severity if given within 1–6 h after exposure	Jonasson et al. (2013b)
15 min	200, 400	Potential Cl-specific markers in BAL (by mass spectroscopy) were chlorohydrins of unsaturated pulmonary surfactant phospholipids; the phosphatidylglycerol chlorohydrins were considered the best biomarkers	Hemstrom et al. (2016)
15 min	300	Postexposure treatment with dexamethasone and *N*-acetyl cysteine, together but not separately, protected against Cl_2-induced AHR and reduced neutrophils in BAL 24 h postexposure. AHR was unaffected by Triptolide, Reparixin, and Rolipram, but Triptolide ↓ inflammation	Wigenstam et al. (2015)

(*Continued*)

TABLE 22.3 (Continued)

Exposure time	Concentration (ppm)	Effect	References
30 min	55–179	The 30-min LC_{50} = 127 (106–152) ppm; ↑ lung weight; tracheal, bronchiolar, and alveolar lesions; regenerative changes seen after 9–10 d	Schlagbauer and Henschler (1967)
3 h	10, 22	Pathology as 30-min exposure; 8/10 died at 10 ppm and 10/10 at 22 ppm	
6 h	10	Similar pathology as after 30-min exposures, 9/10 died	
30 min	187	Preexisting respiratory syncytial virus infection ↑ chlorine-induced lung inflammation, AHR, oxygen desaturation, neutrophil infiltration, and MCP-1, MIP1, IL-10, IFN-, RANTES, and hyaluronan in BAL; type 2 cytokines were unaffected	Song et al. (2015)
30 min	200, 350	Bronchiolitis obliterans (BO) occurred in trachea and large airways within 10 d of exposure to 350 but not 200 ppm in areas where basal progenitor cells were eliminated. BO development included inflammatory cell infiltration by d 2 after exposure, fibroblast infiltration and collagen deposition by d 5, ingrowth of blood vessels by d 7, and lethal airway obstruction by d 9–12	O'Koren et al. (2013)
30 min	400	Hyaluronan low-molecular-weight fragments (L-HA, <300 kDa) and inter-alpha-trypsin-inhibitor (IαI) ↑ in BAL. Airway resistance (methacholine challenge) ↑ 24 h postexposure. AHR ↓ by intratracheal high-MW hyaluronan (H-HA) or antibody against IαI	Lazrak et al. (2015)
30 min	400	Lung deciliation, epithelial sloughing, alveolar inflammation, ↑ airway resistance, ↑ protein and inflammatory cells in BAL; arformoterol (β_2-agonist) given nasally starting 10 min postexposure reduced airway resistance, and improved alveolar clearance	Song et al. (2010, 2011)
30 min	400	In lungs and skin at 1 and/or 6 h postexposure had ↑ IL-6 and TNF-α, ↑ UPR signaling (↑ protein kinase RNA-like endoplasmic reticulum kinase, inositol-requiring enzyme 1a, activating transcription factor 6a), and ↑ expression of hepcidin	Li et al. (2013)
30 min	400	Chlorinated lipids ↑ in the lungs and the plasma (2-chloropalmitic and 2-chlorostearic aldehyde; 2-chloropalmitic and 2-chlorostearic acid, free and esterified) (only parameters evaluated)	Ford et al. (2016)
30 min	400	Had ↑ clotting time, clot formation time, D-dimers, and thrombin–antithrombin complexes in BAL. Treatment with aerosolized heparin ↓ protein levels and inflammatory cells in the BAL at 6 h postexposure	Zarogiannis et al. (2014)
45 min	600		

(*Continued*)

TABLE 22.3 (Continued)

Exposure time	Concentration (ppm)	Effect	References
45 min	600	IM injection of nitrite (10 mg/kg) at 30 or 60 min after Cl_2 exposure improved 24-h survival (20%–50%), with ↓ airway neutrophil accumulation. Females were more sensitive to toxicity and less responsive to nitrite therapy than males	Honavar et al. (2014b)
45 min	600	Most (14/18) died within 72 h of exposure; had labored breathing, lung inflammation, epithelial sloughing; lipid peroxidation; had less mortality (4/18) and toxicity if given ascorbate and deferoxamine (both IM and inhalation) starting 1 h postexposure	Zarogiannis et al. (2011)
60 min	60–160	Neutrophilia, ↑ expression of ARG1, CCL2, RETLNA, IL-1b, and PTGS2 genes, ecog-α, and mannose receptor in BAL fluid, and of activation markers in alveolar macrophages and pulmonary epithelium. Pulmonary elastance was elevated at 48 h	Massa et al. (2014)
60 min	122–193	LC_{50} = 137 (119–159) ppm; had eye and nose irritation, and gasping; survivors had weight loss; liver tissue mottling was seen at necropsy	MacEwen and Vernot (1972)
60 min	228–270	Lung edema and ↑ protein and IgM in BAL; AHR and impaired pulmonary function; rolipram given postexposure (IP or intranasally) inhibited pulmonary edema and airway hyperreactivity	Chang et al. (2012)
60 min	240	IM injection of microencapsulated formulations of rolipram, triptolide, and budesonide ameliorated chlorine-induced acute lung injury (pulmonary edema, AHR, ↑ neutrophils in lung tissue)	Hoyle and Svedsen (2016)
60 min	240	Sloughing of Clara and ciliated cells from tracheal epithelium at d 2; after 2–4 d had cell proliferation in K5- and K14-expressing basal cells; at d 7–10 fibrosis was seen primarily in distal trachea	Musah et al. (2012)
60 min	240	Upregulation of genes related to macrophage function. IM injection with budesonide for 7 d starting 1 h postexposure prevented influx of M2 macrophages, airway fibrosis, and AHR, but large airways had poorly repaired epithelium; same findings at 14 d postexposure	Musah et al. (2019)
60 min	240	Airway epithelial damage and hyperreactivity, neutrophil inflammation, lung edema and dysfunction; mometasone and budesonide (IP, given 1 h postexposure) decreased lung neutrophil levels and lung edema	Chen et al. (2013)

(*Continued*)

TABLE 22.3 (Continued)

Exposure time	Concentration (ppm)	Effect	References
60 min	240	FVB/NJ mice and A/J mice had fibroproliferative lesions in large airways 4 d postexposure, which by d 7 was repaired in A/J mice but fibrosis in FVB/NJ mice, latter had less keratin 5 in basal cells of large airways	Mo et al. (2013)
60 min	240	A/J mice had bronchial epithelium sloughing 1 d postexposure, repaired by d 7 with abnormal cell distribution. Developed (nonbacterial) pneumonitis with ↑ neutrophils and macrophages; had ↑ in chemokines, granulocyte colony-stimulating factor, and VEGF and protein in BAL	Mo et al. (2015)
60 min	1.7–8.8	RD_{50} = 3.5 ppm; appearance of animals was not addressed	Gagnaire et al. (1994)
120 min	2.2–6.6	Respiratory rate ↓ 31%–65% after 60 min, and ~38%–72% after 120 min	
67 min	179–263	Several mice died within 24 h after exposure; had edema, airway epithelial sloughing, and ↑ protein and neutrophils in BAL	Tian et al. (2008)
6 h	9.1 ± 1.02	Marked nasal cavity epithelial degeneration, ulceration, and necrosis, most severe in olfactory mucosa of anterior dorsal meatus	Jiang et al. (1983)
16 h	63	All survived; showed less distress than at higher concentrations	Weedon et al. (1940)
	250, 1000	Lacrimation, dyspnea, convulsions; all died after 8.4 h (250 ppm) and 50 min (1000 ppm); had lesions in lungs and numerous internal organs	
24 h	45	Mean survival time was 7.6–38 h for 40 strains; had lung hemorrhage, alveolar wall thickening, ↑ BAL protein and altered metabolite content	Leikauf et al. (2010, 2012)

↑, increase(d); ↓, decrease(d); *AHR*, airway hyperresponsiveness; *BAL*, bronchoalveolar lavage (fluid); *BW*, body weight(s); *d*, day(s); *IM*, intramuscular; *IP*, intraperitoneal; LC_{50}, the concentration that results in 50% mortality; *mo*, month(s); *min*, minutes; *OE*, olfactory epithelium (nasal); RD_{50}, concentration causing a 50% decrease in the respiration rate; *wk*, week(s); *yr*, year(s).

Single-exposure durations ranged from 2 min to 24 h, and repeat-exposure durations were 3 days to 2 years (1–8 h/day). Older single-exposure studies often evaluated toxicity for a period of days to weeks after exposure to chlorine concentrations extending into the lethal range. Some older studies quantified sensory irritation in rats and mice during a short-term exposure (10–60 min), by determining the concentration that caused a 50% decrease in the inhalation rate (i.e., the RD_{50}). Many recent studies treated animals with near-lethal chlorine concentrations for a short duration (<1 h), followed by sacrifice within hours or a few days. The latter studies were designed to explore aspects of the mechanism of chlorine toxicity, including its progression over time and postexposure tissue repair, as well as the ability of various classes of compounds to mitigate chlorine toxicity (see this Sections 22.4, 22.6, and 22.7).

The respiratory tract was consistently the primary and most sensitive target of toxicity, and similar effects were seen following single and repeated inhalation exposures. The upper respiratory tract was predominantly affected at lower chlorine concentrations, with symptoms such as sensory irritation and nasal lesions. Sensory irritation was manifested as a decreased respiration rate, sneezing,

TABLE 22.4 Animal repeat-exposure chlorine inhalation studies.

Exposure scenario	Concentration (ppm)	Effect	References
Monkeys			
6 h/d, 5 d/wk × 12 mo	0.10 ± 0.03, 0.50 ± 0.10, 2.3 ± 0.4	Eye tearing and reddening during exposure to 2.3 ppm seen after ~6 wk; after 1 yr had conjunctival irritation but no gross eye lesions. Had trace or mild nasal epithelial hyperplasia, loss of cilia and goblet cells (dose-related), with tracheal involvement at 2.3 ppm	Klonne et al. (1987)
Rats			
6 h/d × 3 d 6 h/d × 5 d	9.1 ± 1.02 (TWA)	BW ↓; marked nasal epithelial degeneration, cell exfoliation, ulceration, and necrosis; neutrophil infiltration of epithelium and squamous metaplasia after 5 d; milder lesions in pharynx, trachea, and lungs	Jiang et al. (1983)
6 h/d × 5 d	0.1, 0.5, 1.0 2.5	Nasal inflammation and hyperplasia in squamous and respiratory epithelium (concentration-dependent) but not OE; extended to the nasopharyngeal duct at 2.5 ppm	George et al. (2010), Jarabek et al. (2010)
With 7-d recovery	1.0, 2.5	Hyperplasia (concentration-dependent) in squamous and respiratory epithelium of anterior nasal sections	
6 h/d, 5 d/wk × 2 wk	12	Looked unkempt, wheezed, had swelling around eyes and nares, ↓ BW, and reversible ↑ in lung sulfhydryl level after 3–6 d	Dodd et al. (1980)
6 h/d, 5 d/wk × 6 wk	1	Signs of irritation, mild nasal, tracheal, bronchial, alveolar inflammation	Barrow et al. (1979)
	3	As at 1 ppm; also, lacrimation, conjunctivitis, nasal discharge, liver lesions	
	9	As at 3 ppm; also, gasping, lung rales, emaciation; 3/20 died by 30th exposure; lesions in lungs, liver, kidney, stomach, spleen, and thymus	
6 h/d × 104 wk; 5 d/wk (M) or 3 d/wk (F)	0.4, 1.0, 2.5	BW ↓ in all M and at 1.0 and 2.5 ppm in F after >3 mo; closed eyes at 2.5 ppm in F. All had nasal lesions, most severe in anterior and generally concentration-related (inflammation; OE degeneration; squamous metaplasia; goblet cell epithelial hypertrophy and hyperplasia)	Wolf et al. (1995)
Mice			
8 h/d × 3 d	2.5	BW was 93.2% of initial weight	Schlagbauer and Henschler (1967)
	5	BW was 87.5% of initial weight; lesions in tracheal epithelium and mucosa, bronchioles, and alveoli	
6 h/d × 3 d 6 h/d × 5 d	9.1 ± 1.02	BW ↓; marked nasal lesions (epithelial degeneration, ulceration, necrosis); neutrophil infiltration of epithelium and squamous metaplasia after 5 d; milder lesions in pharynx, larynx, trachea, lungs	Jiang et al. (1983)
6 h/d × 5 d	9.7	Nasal, tracheal, and lung lesions, with anterior to posterior severity gradient (exfoliation, necrosis, inflammation, squamous metaplasia, hyperplasia, bronchiolitis)	Buckley et al. (1984)
6 h/d, 5 d/wk × 104 wk	0.4, 1.0, 2.5	BW ↓ for M at 1.0 and 2.5 ppm and F at 2.5 ppm after >3 mo. All had nasal lesions, most severe in anterior and generally concentration-dependent (septal fenestration; proteinaceous accumulation; epithelial hyperplasia; squamous metaplasia; OE atrophy)	Wolf et al. (1995)

↑, increase(d); ↓, decrease(d); *BW*, body weight(s); *d*, day(s); *F*, female(s); *M*, male(s); *mo*, month(s); *OE*, olfactory epithelium (nasal); *wk*, week(s).

flared nostrils, reddened eyes, lacrimation, salivation, and foamy nasal secretions. Nasal symptoms included inflammation, epithelial necrosis, deciliation, hyperplasia, and metaplasia. At higher chlorine concentrations, the lower respiratory tract was also affected, and manifested as dyspnea, emphysema, bronchopneumonia, pulmonary edema, hemorrhage, and increased protein content and IgM in the bronchoalveolar (BAL) fluid. In many cases, the irritation and pulmonary toxicity were reversible, but at sufficiently high doses, lung toxicity led to permanent changes and/or death. Exposure to near-lethal concentrations caused lesions in organs outside the respiratory tract, including the spleen, thymus, brain, lungs, heart, stomach, intestines, liver, and kidneys. In the single-exposure studies, toxicity was generally influenced more by exposure concentration than exposure duration; this relationship was less clear in the repeat-exposure studies, which were complicated by intermittent periods of nontreatment (e.g., 5 days/week), allowing partial recovery of the chlorine-induced lesions.

No animal studies have linked chlorine exposure with developmental or reproductive toxicity or cancer. Genotoxicity studies with chlorine gas yielded negative results, but mixed results were obtained with the chlorine hydrolysis product hypochlorite (U.S. EPA, 1999; ATSDR, 2010).

22.6 Risk assessment

There are several factors to consider in defining the risk of chlorine inhalation on a diverse human population and in defining levels that are safe for short- and long-term exposures. For example, how do age, gender, and preexisting respiratory conditions such as asthma and AHR affect susceptibility to chlorine toxicity? How do chlorine exposure concentration and duration contribute to chlorine toxicity?

A review of human chlorine inhalation studies led White and Martin (2010) to conclude that there was no basis for considering young subjects more susceptible than adults to chlorine toxicity. Although it was speculated that children might be more susceptible than adults to chlorine toxicity based on reports of illness following several accidental chlorine releases, the actual air chlorine levels and exposure durations of the subjects were unknown (ATSDR, 2010).

Among healthy nonasthmatic adults aged 18−69 years who were exposed to 1.0 ppm chlorine for 15 min, nasal airway resistance, congestion, and irritation increased with age and preexisting allergic rhinitis, independent of gender (Shusterman et al., 2003a,b, 2004). Adults with nonspecific AHR or asthma, or those who smoke, were more susceptible to chlorine respiratory symptoms and pulmonary dysfunction and recovered more slowly after chlorine exposure (Rotman et al., 1983; D'Alessandro et al., 1996; ATSDR, 2010). Studies with mice showed that preexisting respiratory syncytial virus infection increased chlorine-induced lung inflammation and AHR (Song et al., 2015).

The degree to which endpoints such as respiratory tract irritation and mortality are influenced by chlorine exposure concentration or duration have been evaluated using studies that varied both parameters. The concentration−time relationship for many irritants and systemically acting vapors and gases can be described by the equation $C^n \times t = k$ (ten Berge et al., 1986), which is equivalent to Haber's rule ($C \times t = k$) when $n = 1$. A value of $n = 1.9$ was determined for chlorine gas nuisance irritation by ten Berge and Vis van Heemst (1986) using the Anglen (1981) human data. This value of n indicates that exposure concentration is a greater determinant of toxicity than duration. A similar conclusion was drawn by Shusterman et al. (2004) in their analysis of chlorine sensory irritation in a number of controlled human studies. Haber's rule did not adequately capture the type, incidence, and severity of nasal lesions in female rats exposed once to 0.1−10 ppm chlorine for 1−24 h, such that the product of the test concentration and duration ($C \times t$) was constant (Peay et al., 2010). Mice exposed to 100−800 ppm chlorine at a constant $C \times t$ of 100 ppm/h had much greater mortality and lung injury at 800 ppm (0.125 h exposure) than at 100 ppm (1 h exposure) (Hoyle et al., 2010).

Currently, US occupational exposure to chlorine is limited to a ceiling value of 0.5 ppm by NIOSH (REL) and 1 ppm by OSHA (PEL) (NIOSH, 2018). The ACGIH recently lowered its 8-h TLV-TWA from 0.5 to 0.1 ppm (ATSDR, 2010; Kurowski, 2018). These standards reflect human data showing that inhalation of 0.5−1 ppm chlorine, whether once or repeatedly, causes mild upper respiratory tract irritation, which is more severe in individuals with preexisting conditions such as asthma, rhinitis, and AHR (Anglen, 1981; D'Alessandro et al., 1996; Joosting and Verberk, 1974; Rotman et al., 1983; Shusterman et al., 1998, 2003a,b, 2004). The human studies also served as the basis for guidelines to prevent adverse but nondisabling effects from a single 10-min to 24-h inhalation exposure. The acute exposure guideline levels (AEGLs) limit chlorine air concentration to 0.5 ppm for 10−480-min exposures (Talmage, 2004). The provisional advisory levels (PALs) limit chlorine levels to 0.096 ppm for a 24-h exposure (Milanez et al., 2014). Additional AEGL and PAL values were developed for chlorine that are associated with serious irreversible effects or lethality (Talmage, 2004; Milanez et al., 2014). Development of sensitive methods to detect and quantify chlorine adducts 3-chlorotyrosine and 3,5-dichlorotyrosine in human blood (Crow et al., 2016) provides another tool for accurate

exposure assessment to use in chlorine human health and risk assessments.

22.7 Treatment

A chlorine-specific antidote has not been developed, although studies are underway to find treatments that are more specific and effective. Current medical treatment for chlorine inhalation is largely nonspecific and designed to alleviate the patient's symptoms (Jones et al, 2010; Govier and Coulson, 2018). The most commonly prescribed medications for people hospitalized following chlorine gas release in the 2005 train accident in Graniteville, South Carolina, were inhaled β-agonists, ipratropium bromide (an anticholinergic), and inhaled and oral corticosteroids (Van Sickle et al., 2009). Routine treatment after Cl_2 exposure includes humidified oxygen, inhaled beta-adrenergic agents if there is evidence of airway obstruction, and copious water irrigation of eyes if ocular irritation is present (White and Martin, 2010). Questions have been raised regarding the safety of oxygen treatment, as rat studies suggested that although it increased short-term survival, it exacerbated respiratory failure (Okponyia et al., 2018). Respiratory peak flow is measured in patients with mild or greater symptoms, and patients with moderate or severe effects should have a chest X-ray (IPCS, 2008). All symptomatic patients should be observed for 8−24 h for delayed pulmonary edema, which can be treated with positive airway pressure or endotracheal intubation and mechanical ventilation in extreme cases (Traub, 2006; IPCS, 2008). The majority of individuals exposed to mild to moderate chlorine levels see resolution of their symptoms in 3−5 days and recover normal pulmonary function within several months, although injury of the distal airways can lead to persistent respiratory symptoms and lung-function decrements (Jones et al., 2010; Hoyle and Svedsen, 2016; Govier and Coulson, 2018).

Various compounds have been tested in animals in the quest to develop an effective chlorine-specific treatment based on its mechanism of action (Tables 22.3 and 22.4). Sodium bicarbonate can theoretically neutralize the HCl and HOCl formed in the respiratory tract from Cl_2 hydrolysis, although its efficacy is questionable (Jones et al, 2010). Nebulized sodium bicarbonate increased the severity of pulmonary injury in rats at 30 min postexposure but had no effect 45 days after a 5-min exposure to 155 ppm chlorine (Al et al., 2010). Mice given a single intramuscular (i.m.) injection of nitrite (10 mg/kg) at 30 or 60 min after Cl_2 exposure had improved 24-h survival (from ∼20% to 50%), with decreased neutrophil accumulation in the airways (Honavar et al., 2014b). Similarly, rabbits given i.m. nitrite (1 or 10 mg/kg) at 30 min after Cl_2 exposure had up to 50% less protein and neutrophil accumulation in the airways and no mortality (∼35% mortality over 18 h in controls) (Honavar et al., 2017). The authors propose i.m. nitrite treatment for postexposure use in human mass-casualty scenarios. Yadav et al. (2011) showed that rats given intraperitoneal (i.p.) injections of sodium nitrite 10 min to 6 h after chlorine gas exposure had significantly less lung airway and epithelial injury than untreated rats.

Other evaluated compounds include corticosteroids such as mometasone, budesonide, and dexamethasone (Chen et al., 2013; Jonasson et al., 2013b; Hoyle and Svedsen, 2016; Musah et al., 2019), which are used to reduce the inflammatory response and lung scarring; the antioxidants dimethylthiourea, ascorbic acid, *N*-acetyl-L-cysteine, deferoxamine, and AEOL-10150 (Leustik et al., 2008; McGovern et al., 2010, 2011; Zarogiannis et al., 2011; Wigenstam et al., 2015, 2016; Zhang et al., 2018); the type 4 phosphodiesterase inhibitor rolipram (Chang et al., 2012; Hoyle and Svedsen, 2016), the β2-agonist arformoterol (Song et al., 2011); and the plant antiinflammatory product triptolide (Hoyle and Svedsen, 2016). Mice given i.p. doses of dexamethasone and *N*-acetyl cysteine, together but not separately, 1 h after chlorine exposure, were protected against Cl_2-induced AHR and neutrophilia (Wigenstam et al., 2015). Inhibitors of the vanilloid-type transient receptor potential channel TRPV4 decreased vascular leakage and improved blood oxygenation after chlorine gas exposure in mice, but their use in treating chlorine toxicity is limited by their concomitant inhibition of the immune response to pathogen-provoked injury (Morty and Kuebler, 2014). A recent review of animal models developed for treatment of acute chlorine injury included R-107, which is a nitric oxide donor, peroxynitrite modulator, and superoxide scavenger, in an ovine i.m. treatment model (Summerhill et al., 2017).

22.8 Concluding remarks and future directions

Chlorine is widely used in manufacturing and as a disinfectant and bleach, despite its toxicity and potential for nefarious use. Chlorine is a contact irritant and its toxicity is believed to be due to its oxidant properties and to be mediated by the hypochlorite ion, although the precise mechanism is unknown. The chlorine exposure concentrations and durations, and dose-response, associated with various degrees of toxicity have been evaluated in humans and laboratory animals. Upon chlorine inhalation, the respiratory tract is the predominant and most sensitive target organ in all species. The irritation and pulmonary toxicity resulting from exposure to low chlorine concentrations are in many cases reversible, but exposures to sufficiently high concentrations can led to permanent

lung lesions and to injury of other internal organs, and can be lethal. Treatment for chlorine inhalation is largely supportive and nonspecific, although a plethora of studies have attempted to elucidate chlorine's mechanism of action and find treatments to mitigate its toxic effects. To assist in providing guidance for decision response and risk mitigation after accidental chlorine release, computer simulations have been developed to predict the spread of chlorine gas, indoor−outdoor air exchange, and the potential number of people injured within a given radius. Many questions remain unanswered regarding the mechanism of chlorine toxicity and the optimal treatment for a population following an unexpected chlorine release.

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Chapter 23

Phosgene

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23.1 Introduction

Phosgene is a colorless gas at ambient temperature and pressure. Its odor has been described as similar to newly mown hay (Leonardos et al., 1968). This mild odor and the weak acute irritant properties, however, provide little warning of its presence (Lipsett et al., 1994).

Phosgene is manufactured from a reaction of carbon monoxide and chlorine gas in the presence of activated charcoal. Phosgene is used in the manufacture of isocyanates, polycarbonates, pesticides, dyes, and pharmaceuticals. Manufacture of phosgene in the United States is almost entirely captive in that more than 99% is used in the manufacture of other chemicals within a plant boundary (US EPA, 2003). The odor threshold is between 0.5 and 1.5 ppm (NIOSH, 1976); unfortunately, the odor threshold is inadequate to protect against toxic inhalant exposure because damage to the deep respiratory tract can take place at lower concentrations (Sidell et al., 1997). Phosgene has an odor safety classification rating of "E," which indicates that fewer than 10% of attentive persons can detect the threshold limit value (Amoore and Hautala, 1983).

Inhalation is the most important route of exposure for phosgene. Because of its mild upper respiratory, eye, and skin irritancy, and a mildly pleasant odor, an exposed victim may not actively seek an avenue of escape before lower respiratory damage has occurred (Currie et al., 1987a,b; Lipsett et al., 1994). Small amounts of phosgene can be irritating to the eyes (lacrimation) and throat (coughing) as phosgene undergoes hydrolysis to create hydrochloric acid, which acts as an irritant. Far more dangerous, however, is the pulmonary edema that can develop after a latent period of 1−24 h after exposure. Exercise can increase the extent of pulmonary edema (Marrs et al., 1996). If severe clinical signs are not present after 48 h, then the chances of full recovery are excellent. Physical and chemical properties of phosgene are summarized in Table 23.1.

23.2 Background

Phosgene was first made in 1812 and was used as a chemical warfare agent in WWI. Because of its higher density compared with air, phosgene gas can accumulate in low-lying areas; thus, concentrated pockets within war trenches caused significant exposure during WWI. German troops in WWI used phosgene gas as a chemical warfare agent against British troops. Possibly up to 85% of deaths in WWI were due to chemical exposure (Ministry of Defense, 1987). Sandall (1922) examined 83 British soldiers 3 years after phosgene exposure. Shortness of breath on exertion (70%), cough with expectoration (54%), tight feeling in the chest (25%), sporadic giddiness (14%), and nausea (12%) were the most frequently reported symptoms. No physical lung abnormalities were noted in 53% of the men.

The concept of a "death product" was introduced by Haber to explain the relationship between the extent of exposure to phosgene and death (Haber, 1924). According to "Haber's law," the biological effect of phosgene is directly proportional to the exposure expressed as the product of the atmospheric concentration (C) and the time of exposure (T) or $CT = k$, where k can be death, pulmonary edema, or other biological effects of phosgene exposure (US EPA, 1986). Haber's law has subsequently been shown by other investigators to be valid for both nonlethal and lethal effects within certain limits. There appears to be little species variability with regard to lethality between rats, mice, and guinea pigs, and the $CT = k$ relationship appears to be generally valid (although at very high or very low concentrations or exposure times so short that the animal can hold its breath, the $CT = k$ relationship may not be relevant).

23.3 Toxicokinetics

After inhalation exposure, a small portion of phosgene hydrolyzes to hydrochloric acid (HCl) and carbon dioxide

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TABLE 23.1 Physical and chemical data.

Parameter	Data	References
Synonyms	Carbonyl chloride, carbon oxychloride, carbonic dichloride, chloroformyl chloride	US EPA (1986) and Lipsett et al. (1994)
Chemical formula	$COCL_2$	Lipsett et al. (1994)
Molecular weight	98.92	Lipsett et al. (1994)
CAS registry No.	75-44-5	Lipsett et al. (1994)
Physical state	Colorless gas at room temperature	Budavari et al. (1989)
Odor threshold	0.5−1.5 ppm	NIOSH (1976)
Odor description	Pleasant, like newly mown hay	Dunlap (2001)
Vapor pressure	1215 mmHg at 20°C	Budavari et al. (1989)
Vapor density	3.5 (air = 1)	NIOSH (2010)
Specific gravity	1.92 (water = 1)	CRC Handbook (2013)
Melting/boiling/flash point	−118°C/8.2°C/nonflammable	NIOSH (2010)
Water solubility	Slightly soluble in water, decomposes rapidly ($t_{½}$ = 0.26 s)	US EPA (2003); NIOSH (2010)
Reactivity	Reacts with alcohols, alkalis, ammonia, and copper	NIOSH (2010)
Conversion factors in air	1 ppm = 4.1 mg/m^3 1 mg/m^3 = 0.25 ppm	NIOSH (2010)

(CO_2) in the mucous coating of the upper respiratory tract (Diller, 1985), but in the moist atmosphere of the terminal spaces of the lungs more extensive hydrolysis is thought to occur (Beard, 1982). Although phosgene is only slightly soluble in water, once in solution it rapidly hydrolyzes to HCl and CO_2. However, phosgene reacts even faster with other functional groups such as amino, hydroxyl, and sulfhydryl groups (Diller, 1985; Jaskot et al., 1991). Because of the affinity for lung tissue and the hydrolysis and acylation that take place in the pulmonary system, very little, if any, phosgene is dispersed to other locations in the body.

23.4 Mechanism of action

The toxicity of phosgene is attributable to both hydrolysis and acylation, with the latter being most important. Diller (1985) accumulated data and described the human clinical signs associated with phosgene exposure. Phosgene inhaled at concentrations of more than 1 ppm triggers a transient vagal reflex and causes shallow, rapid respiration with a decrease in respiratory volume and capacity, a decrease in arterial oxygen partial pressure, and bradycardia. The intensity varies greatly between individuals. Phosgene at more than 3 ppm becomes moderately irritating to the eyes and upper airways (cough) as some of the phosgene undergoes hydrolysis, producing HCl.

The acylation reaction of phosgene with nucleophiles, such as amino, hydroxyl, and sulfhydryl groups, also occurs rapidly, causing lipid and protein denaturation, irreversible membrane changes, and disruption of enzymatic function. These acylation effects observed at exposures more than 30 ppm/min produce pulmonary edema as the blood−air barrier becomes more permeable to blood plasma after a clinical latent period (1−24 h). This is called the clinical edema phase, which is characterized by increasing inefficiency of gas exchange as more defects in the blood−air barrier occur, allowing more accumulation of a protein-rich fluid. Clinical signs in this phase are labored breathing and a frothy expectorant. Progression usually results in death from paralysis of the respiratory center due to anoxemia with secondary cessation of heart function. If anoxemia is controlled, then circulatory shock may still occur. At very high concentrations (>200 ppm), phosgene may cause death within a few minutes from "acute cor pulmonale" (acute overdistension of the right heart), often before pulmonary edema can develop.

Phosgene depletes lung glutathione, whereas glutathione reductase and superoxide dismutase increase as a result of the lung's response to injury. On exposure to phosgene, cellular glycolysis, oxygen uptake, intracellular ATP, and cyclic AMP are decreased and associated with increased permeability of pulmonary vessels, leading to

pulmonary edema. Phosgene exposure also causes increased lipid peroxidation and leukotriene synthesis, with no change in cyclooxygenase metabolism (Borak and Diller, 2001).

In rats, the first event observed following high but sublethal acute exposure to phosgene was the stimulation of alveolar nociceptive vagal receptors; the nociceptive reflexes, with changes in cardiopulmonary function, resembled typical patterns of alveolar irritation (Luo et al., 2014). The degree and time-course of pulmonary injury were reflected best by exhaled nitric oxide/exhaled CO_2 ratios, hemoconcentration, and protein in bronchoalveolar lavage (BAL) fluid. Significant changes in cardiopulmonary function, ventilation:perfusion imbalances, and progressive pulmonary edema and phospholipoproteinosis were noted. Phosgene-induced acute lung injury showed evidence of persistent periods of apnea, bradycardia, and shifts of vascular fluid from peripheral to pulmonary circulation. Carbon dioxide in expired gas was suggestive of increased ventilation dead space and appeared to be predictive of developing lung edema. Treatment with the iNOS inhibitor aminoguanidine aerosol by inhalation decreased the severity of phosgene-induced ALI when applied at low dose-rates. Symptomatic treatment protocols were inferior to mechanistic treatment methods (Li and Pauluhn, 2017).

23.5 Toxicity

23.5.1 Human

23.5.1.1 Noncancer

Reports of human phosgene poisoning present a relatively consistent set of clinical effects and sequelae (Delephine, 1922; Hegler, 1928; Galdston et al., 1947a,b; Herzog and Pletscher, 1955; Everett and Overholt, 1968; Henschler, 1971; Stavrakis, 1971; Diller et al., 1979; Bradley and Unger, 1982; Misra et al., 1985; Regan, 1985; Wells, 1985; Cordasco et al., 1986; Kaerkes, 1992; Hardison et al., 2014). After acute phosgene exposure, brief (20 min) ocular and throat irritation, cough, nausea and vomiting, and dizziness are experienced, followed by a period (24 h) of apparent well-being. After this clinical latent phase, cough accompanied by expectoration, a sensation of pain or tightness of the chest, shortness of breath, and a choking sensation are experienced. Clinical findings may include hemoconcentration, leukocytosis, rales, and pulmonary edema. After recovery, rapid and shallow breathing, shortness of breath on exertion, and a sense of decreased physical fitness may persist for months. Pulmonary emphysema may occur with repeated exposure to phosgene.

Diller and Zante (1982) identified ocular irritation, throat irritation, and cough as acute irritating effects of phosgene. In a follow-up analysis, Borak and Diller (2001) also performed an extensive literature review concerning human phosgene exposure and concluded the following: the smell has no warning property; immediate irritation is not prognostic; and pulmonary edema can appear several hours after exposure and the length of clinical latency can be used as a prognostic indicator (i.e., the shorter the time to effects, the worse the prognosis). The data also show that although concentration is the primary driver for the onset and severity of symptoms, duration of exposure also plays a role. Information synthesized from these reviews is presented in Table 23.2.

23.5.1.2 Cancer

Epidemiology studies have shown no increase in cancer in workers exposed to phosgene compared with controls. Polednak (1980) and Polednak and Hollis (1985) examined a cohort of chemical workers exposed to chronic low levels of phosgene as well as daily exposures of more than 1 ppm. Approximately 35 years after exposure to phosgene, no increase in overall mortality or mortality from cancer or respiratory disease was noted.

23.5.2 Animal

23.5.2.1 Noncancer

Animal studies with phosgene show a steep concentration−response curve for pulmonary edema and mortality. Acute animal studies also indicate little species variability because rats, mice, sheep, pigs, and dogs exposed had development of similar clinical signs (dyspnea, labored breathing, and pulmonary edema after a latent period) and histopathological lesions in the lungs. Although there are no chronic animal data, subchronic studies indicate little accumulation of phosgene or increased severity of lesions with continuous exposure. BAL fluid analysis may be used to assess pulmonary edema/lung injury after acute inhalation exposure to phosgene (Pauluhn et al., 2007). Maximum protein concentrations in BAL fluid typically occur within 1 day after exposure, followed by a latency period up to 15 h. For acute exposures, the *CT* relationship is constant over a wide range of concentrations. However, after noncontinuous and repeated exposure, increased tolerance to subsequent exposures is observed. Although limited, the longer term data indicate that effects do not increase in severity over time but do increase with increased concentration, suggesting that chronic toxicity is dependent on an acute pulmonary threshold dose.

A comparison of BAL fluid constituents from acute inhalation studies in rats and dogs suggests that dogs are three to four times less sensitive to phosgene than rats (Pauluhn et al., 2007). Li and Pauluhn (2019) determined

TABLE 23.2 Summary of major signs and symptoms of phosgene inhalation exposure in humans.

Exposure			Acute effect	Time to onset of pulmonary edema (h)	Time to death
Concentration (ppm)	Duration	$C \times T$ product (ppm min)			
3	"Acute"	–	Throat irritation	–	–
4	"Acute"	–	Ocular irritation	–	–
4.8	"Acute"	–	Cough	–	–
1	20 min	20	–	–	–
5	5 min	25	–	–	–
1	150 min	150	–	10	–
50	5 min	250	–	5	–
100	5 min	500	–	3	24 h
1.3	400 min	520	–	7	30 h
300	2 min	600	–	–	Minutes

Source: Data from Diller, W.F., Zante, R., 1982. Dosis-wirkungs-beziehungen bei phosgen-einwirkung auf mensch und tier. Zbl. Arbeitsmed. 32, 360–368 and Borak, J., Diller, W.F., 2001. Phosgene exposure: mechanisms of injury and treatment strategies. J. Occup. Environ. Med. 43, 110–119.

that increased lung weight in rats and dogs with phosgene-induced acute lung injury corresponded well with excess extravascular lung water in humans. Thus, this endpoint was considered the biomarker of choice for scaling lung edema from experimental animals to humans. The predictive value of data from dogs was better than that of rats, suggesting that canine data should be given preference over rodent data when scaling to the human. In rats, increased BAL protein may lead to overestimation of the phosgene edema potency due to secreted protein into airways. Thus, caution is advised when using BAL protein in isolation as a surrogate endpoint of pulmonary edema.

A summary of selected lethal and nonlethal animal toxicity studies is presented in Tables 23.3 and 23.4, respectively.

23.5.2.2 Animal cancer

A study by Selgrade et al. (1989) showed that exposure to very low levels of phosgene enhances the susceptibility of mice to lung tumor formation. Female C57BL/6 mice were exposed for 4 h to 0.01 ($N = 13$), 0.025 ($N = 28$), or 0.05 ppm phosgene ($N = 35$) and injected intravenously with syngeneic B16 melanoma cells the next day. Controls were injected with tumor cells and exposed to air. The lungs were removed 2–3 weeks after tumor cell injection and the tumors were counted. Compared with controls, there was a statistically significant ($P < .05$) increase in the number of B16 melanoma tumors in the lungs of mice treated with 0.025 or 0.05 ppm phosgene. Exposure to 0.025 ppm was considered the lowest observed effect level. Extending the exposure time from 4 to 8 h did not alter the susceptibility to B16 tumors at 0.01 ppm.

23.6 Risk assessment

Many inhalation regulatory and guideline levels have been derived for phosgene. These values are summarized in Table 23.5, and the definitions and basis for the values are described in the footnotes to Table 23.5.

23.7 Treatment

Treatments that have been proposed to prevent pulmonary edema in exposed persons include sedation, steroids, ibuprofen, *N*-acetylcysteine, B2-adrenergic agonists, aminophylline/theophylline, leukotriene agonists, and positive pressure airway ventilation. However, there is no known antidote for phosgene poisoning, and although animal studies suggest that these treatments may be effective, no clinical data are available to verify efficacy in humans (Borak and Diller, 2001; Grainge and Rice, 2010; ACC, 2013). Asymptomatic individuals exposed to phosgene should be observed and evaluated to determine if symptoms develop. Vital signs and lung auscultation should be

TABLE 23.3 Summary of lethal animal inhalation studies with phosgene.

Concentration (ppm)	Time (min)	Species	Effect	References
Various	30	Dog	LC_{50} = 66 ppm	Boyland et al. (1946)
Various	30	Dog	LC_{50} = 61–70 ppm	Underhill (1920)
Various	60	Dog	LC_{50} = 42 ppm	Boyland et al. (1946)
Various	8	Rat	LC_{50} = 92 ppm	Boyland et al. (1946)
12, 37, 75, 80, 88, 93, or 106	10	Rat	LC_{50} = 82 ppm	Zwart et al. (1990)
41, 44, 52, or 61	10	Rat	LC_{50} = 62 ppm	Pauluhn (2006a)
12, 15, 16, 17, or 25	30	Rat	LC_{50} = 21 ppm	Zwart et al. (1990)
12, 13, 17, or 22	30	Rat	LC_{50} = 13.5 ppm	Pauluhn (2006a)
Various	32	Rat	LC_{50} = 17 ppm	Boyland et al. (1946)
6.4, 8.8, 9.0, or 12	60	Rat	LC_{50} = 12 ppm	Zwart et al. (1990)
7.3, 9.6, or 12	60	Rat	LC_{50} = 7.7 ppm	Pauluhn (2006a)
Various	64	Rat	LC_{50} = 11 ppm	Boyland et al. (1946)
2.2 or 2.7	240	Rat	LC_{50} = 2.1 ppm	Pauluhn (2006a)
10, 15, 25, 35, 50, 70, or 90	5	Mouse	LC_{50} = 33 ppm	Kawai (1973)
Various	8	Mouse	LC_{50} = 77 ppm	Boyland et al. (1946)
12, 37, 75, 80, 88, 93, or 106	10	Mouse	LC_{50} = 79 (m) and 60 (f) ppm	Zwart et al. (1990)
12, 15, 16, 17, or 25	30	Mouse	LC_{50} = 19 (m) and 11.5 (f) ppm	Zwart et al. (1990)
1.0, 2.0, 3.0, 6.0, 9.0, or 13.5	30	Mouse	LC_{50} = 5.1 ppm	Kawai (1973)
Various	32	Mouse	LC_{50} = 15 ppm	Boyland et al. (1946)
6.4, 8.8, 9.0, or 12	60	Mouse	LC_{50} = 9.5 (m) and 5.0 (f) ppm	Zwart et al. (1990)
Various	64	Mouse	LC_{50} = 7 ppm	Boyland et al. (1946)
Various	8	Guinea pig	LC_{50} = 43 ppm	Boyland et al. (1946)
Various	32	Guinea pig	LC_{50} = 13 ppm	Boyland et al. (1946)
Various	64	Guinea pig	LC_{50} = 11 ppm	Boyland et al. (1946)

evaluated every 30 min and serial chest X-rays should be performed starting 2 h after exposure. If no clinical signs occur and no signs of pulmonary abnormalities are detected on the X-ray after 8 h, then patients may be discharged. If no X-ray is available, then patients should be observed for 24 h after exposure. If signs develop, then patients should be treated. Therapy for the cardiogenic pulmonary edema may include positive airway pressure and monitoring (endotracheal intubation and mechanical ventilation with high oxygen concentrations), steroids, theophylline, diuretics, and antibiotics (in patients who develop bacterial pulmonary infections) (Borak and Diller, 2001).

As discussed in Section 23.4, Li and Pauluhn (2017) show that mechanistic treatment protocols are superior to symptomatic treatment protocols with regard to acute lung injury in rats.

23.8 Concluding remarks and future directions

Phosgene is a colorless gas at ambient temperature and pressure, and inhalation is the most important route of exposure for phosgene. The odor of phosgene has been described as similar to newly mown hay. Because of its mild upper respiratory, eye, and skin irritancy, and mildly pleasant odor, an exposed victim may not actively seek escape before lower respiratory damage has occurred (Currie et al., 1987a,b; Lipsett et al., 1994). Pulmonary edema is the cause of death after a clinical latent period of 24 h (Franch and Hatch, 1986). Phosgene exhibits a steep concentration–response curve and little species variability with regard to lethality. Data (Pauluhn, 2006a,b,c; Pauluhn et al., 2007) suggest that with regard to physiology of the respiratory tract and acinar structure of the

TABLE 23.4 Summary of nonlethal animal inhalation studies with phosgene.

Concentration (ppm)	Time	Species	Effect	References
0 or 60	10 min	Pig	LOAEL ≥ 60 ppm, based on increased lung wet weight, mortality	Brown et al. (2002)
137, 244, 435, or 773	10 min	Sheep	LOAEL ≥ 137 ppm based on pulmonary edema, shallow breathing	Keeler et al. (1990a)
0 or 490 to 611	10 min	Sheep	LOAEL ≥ 490 ppm based on lung edema	Keeler et al. (1990b)
0 or 8	20 min	Mouse	LOAEL ≥ 8 ppm, based on acidosis, clinical signs and ↓body wt, ↑lung ww/dw	Sciuto et al. (2001)
0 or 22	20 min	Mouse, rat, and guinea pig	LOAEL ≥ 22 ppm, based on ↑LFP	Sciuto (1998)
0, 2.1, 4.3, or 8.8	30 min	Dog	LOAEL = 4.3 ppm, based on increased PMNs in BAL fluid NOAEL = 2.1 ppm	Pauluhn (2006c)
0.2, 0.5, 1.0, 2.0, or 4.0	30 min	Rat	LOAEL = 2.0 ppm, based on clinical signs and ↓body wt, ↑LFP NOAEL = 1.0 ppm	Pauluhn (2006b)
0, 0.05, 0.1, 0.2, 0.4, or 1.0	240 min	Rat	LOAEL = 0.2 ppm, based on based on clinical signs and ↓body wt, ↑LFP NOAEL = 0.1 ppm	Pauluhn (2006b)
0, 0.1, 0.5, or 1.0	240 min	Rat	LOAEL = 0.5 ppm based on decrease in NK cell activity NOAEL = 0.1 ppm	Burleson and Keyes (1989)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↓body wt, ↑lung wts	Ehrlich et al. (1989)
0 or 0.5	240 min	Rat	LOAEL ≥ 0.5 ppm based on ↑LFP and lung wts	Jaskot et al. (1989)
0, 0.25, or 0.5	240 min	Guinea pig	LOAEL ≥ 0.25 ppm based on ↑LFP	Slade et al. (1989)
0.1–0.5	240 min	Mouse	LOAEL = 0.15 ppm based on ↑phenobarbital induced sleeping times NOAEL = 0.10	Illing et al. (1988)
0, 0.125, 0.25, 0.5, or 1.0	240 min	Rat	LOAEL = 0.25 ppm based on ↑PMNs in lavage fluid NOAEL = 0.125 ppm	Currie et al. (1987a)
0, 0.05, 0.125, 0.25, 0.5, or 1.0	240 min	Rat	LOAEL ≥ 0.05 ppm based on ↓ATP in lungs	Currie et al. (1987b)
0, 0.1, 0.2, 0.5, or 1.0	240 min	Rat, mouse, and hamster	LOAEL = 0.2 ppm based on ↑LFP NOAEL = 0.1 ppm	Hatch et al. (1986)
0, 0.1, 0.2, 0.5, or 1.0	240 min	Rabbit and guinea pig	LOAEL = 0.5 ppm based on ↑LFP NOAEL = 0.2 ppm	Hatch et al. (1986)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↓body wt, ↑lung wts	Franch and Hatch (1986)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↑pulmonary edema	Frosolono and Currie (1985)

(*Continued*)

TABLE 23.4 (Continued)

Concentration (ppm)	Time	Species	Effect	References
0 or 1.0	420 min	Rat	LOAEL ≥ 1.0 ppm based on ↑lung wts	Franch and Hatch (1986)
0.125 or 0.25	4 h/day, 5 days/week for 17 days	Rat	LOAEL = 0.25 ppm based on ↑lung wts and ↑NPSH and G6PD activity NOAEL = 0.125 ppm	Franch and Hatch (1986)
0, 0.1, 0.2, 0.5, or 1.0	0.1 ppm for 5 days/week; 0.2 ppm for 5 days/week; 0.5 ppm for 2 days/week; 1.0 ppm for 1 day/week. All exposed 6 h/day for up to 12 weeks	Rat	LOAEL = 0.1 ppm based on reversible lung histopathology; ↑lung displacement volume NOAEL = none	Kodavanti and Costa (1997)
0, 0.1, 0.2, or 0.5	0.1 and 0.2 ppm for 5 days/week; 0.5 ppm for 2 days/week. All exposed 6 h/day for up to 12 weeks	Rat	LOAEL = 0.1 ppm based on decreased bacterial clearance after infection with *Streptococcus zooepidemicus* NOAEL = none	Selgrade et al. (1995)

BAL, Bronchiolar lavage; *LFP*, lavage fluid protein.

TABLE 23.5 Inhalation standards and guidelines for phosgene.

Guideline	Exposure duration				
	10 min	**30 min**	**1 h**	**4 h**	**8 h**
AEGL-1[a]	NA	NA	NA	NA	NA
AEGL-2[a]	0.60 ppm	0.60 ppm	0.30 ppm	0.08 ppm	0.04 ppm
AEGL-3[a]	3.6 ppm	1.5 ppm	0.75 ppm	0.20 ppm	0.09 ppm
ERPG-1[b]			NA		
ERPG-2[b]			0.5 ppm		
ERPG-3[b]			1.5 ppm		
EEGL (NRC)[c]			0.2 ppm		0.02 ppm (24 h)
NIOSH IDLH[d]	2 ppm				
NIOSH STEL[e]	0.2 ppm (15 min ceiling)				
NIOSH REL[e]					0.1 ppm (10 h)
OSHA PEL-TWA[f]					0.1 ppm
ACGIH TLV[g]					0.1 ppm
MAK (Germany)[h]					0.1 ppm
MAC (Netherlands)[i]					0.1 ppm
RfC[j]					7.33×10^{-5} ppm
	Exposure duration				
	24 h		**30 days**	**90 days**	**2 years**
PAL 1[k]	0.0017 ppm		0.0006 ppm	0.0006 ppm	0.0006 ppm
PAL 2[k]	0.0033		0.0012 ppm	0.0012 ppm	0.0012 ppm
PAL 3[k]	0.022		NA	NA	NA

[a]*AEGL (acute exposure guideline levels) (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels (AEGL-1, AEGL-2, and AEGL-3) are developed for each of five exposure periods (10 and 30 min, 1, 4, and 8 h) and are distinguished by varying degrees of severity of toxic effects. The three AEGLs tiers are defined as follows. AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. The AEGL-1 is not recommended for phosgene because the odor threshold is at the concentration approaching AEGL-2 values, and odor cannot be used as a warning. AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. The AEGL-2 for phosgene is based on chemical pneumonia in rats (Gross et al., 1965). AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 is based on concentrations causing no death in rats or mice (Zwart et al., 1990).*

[b]ERPG *[*Emergency Response Planning Guidelines, American Industrial Hygiene Association *(AIHA, 2016)]. The ERPG-1 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing other than mild, transient adverse health effects or without perceiving a clearly defined objectionable odor. The ERPG-1 for phosgene is not derived. The ERPG-2 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing irreversible or other serious health effects or symptoms that could impair an individual's ability to take protection action. The ERPG-3 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing life-threatening health effects.*

[c]EEGL *[*Emergency Exposure Guidance Levels, National Research Council *(NRC, 1985)]. The EEGL is the concentration of contaminants that can cause discomfort or other evidence of irritation or intoxication in or around the workplace, but avoids death, other severe acute effects, and long-term or chronic injury. The EEGL for phosgene is based on the "most relevant animal exposure studies (Rinehart and Hatch, 1964; Gross et al., 1965)" and studies suggesting that animals do not tolerate phosgene at 0.2 ppm administered 5 h/day for 5 days (Cameron and Foss, 1941; Cameron et al., 1942).*

[d]IDLH *[*Immediately Dangerous to Life and Health, National Institute of Occupational Safety and Health*(NIOSH, 2010)] represents the maximum concentration from which one could escape within 30 min without any escape-impairing symptoms, or any irreversible health effects. The IDLH for phosgene is based on acute inhalation toxicity data in humans (Diller, 1978).*

[e]*NIOSH REL-STEL (Recommended Exposure Limits—Short Term Exposure Limit) (NIOSH, 2010) is defined as analogous to the ACGIH TLV-TWA.*

[f]OSHA PEL-TWA (Occupational Health and Safety Administration, Permissible Exposure Limits—Time Weighted Average) *(NIOSH, 2010) is defined as analogous to the ACGIH-TLV-TWA, but is for exposures of no more than 10 h/day, 40 h/week.*

[g]ACGIH TLV-TWA (American Conference of Governmental Industrial Hygienists, Threshold Limit Value—Time Weighted Average) *(ACGIH, 2013) is the time-weighted average concentration for a normal 8 h workday and a 40 h workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect. The ACGIH TLV-TWA was derived from the marked potential for pulmonary irritation after exposure to phosgene at concentrations greater than 0.1 ppm; this conclusion was based on two studies. Gross et al. (1965) found that phosgene at 0.5 ppm for 2 h caused some pathologic changes in the lungs of rats and Cameron et al. (1942) found that 0.2 ppm, 5 days/week for 5 consecutive days caused pulmonary edema in 41% of animals exposed (goats, cats, rabbits, guinea pigs, rats, and mice).*

[h]MAK [Maximale Argeitsplatzkonzentration (Maximum Workplace Concentration)*]* *Deutsche Forschungsgemeinschaft (German Research Association) 2017 is defined as analogous to the ACGIH-TLV-TWA.*

[i]MAC [Maximaal Aanvaaarde Concentratie (Maximal Accepted Concentration)]. *SDU Uitgevers (under the auspices of the Ministry of Social Affairs and Employment), The Hague, The Netherlands 2012 is defined as analogous to the ACGIH-TLV-TWA.*

[j]US EPA (US Environmental Protection Agency Reference Concentration) *(US EPA, 2005). The RfC is an estimate of a continuous inhalation exposure concentration to people (including sensitive subgroups) that is likely to be without risk of deleterious effects during a lifetime. The RfC was developed by using incidence of the lung histopathological findings from Kodavanti and Costa (1997) in a bench-mark dose analysis.*

[k]PAL (provisional advisory levels) *(Glass et al., 2008). Provisional advisory levels represent exposure limits for the general public applicable to emergency situations. Three levels (PAL 1, PAL 2, and PAL 3), distinguished by the degree of severity of toxic effects, are developed for up to 24-h, 30-day, 90-day, and 2-year durations of potential drinking water and inhalation exposures. The PALs have not been promulgated nor have they been formally issued as regulatory guidance. They are intended to be used at the discretion of risk managers in emergency situations when site-specific risk assessments are not available.* PAL 1 *represents the assumed continuous exposure concentration of a chemical in air or drinking water above which changes from baseline of specific biomarkers or physiological responses could have adverse health effects in the general population. Concentrations at or below PAL 1 are not expected to be associated with adverse health effects. Increasingly greater concentrations above the PAL 1 value could cause progressively harmful effects in the general population, including all ages and sensitive subpopulations.* PAL 2 *represents the assumed continuous exposure concentration of a chemical in air or drinking water above which serious, irreversible, or escape-impairing effects could result. Increasingly greater concentrations above the PAL 2 value could cause progressively harmful effects in the general population, including all ages and sensitive subpopulations.* PAL 3 *represents the assumed continuous exposure concentration of a chemical in air or drinking water above which lethality in the general population, including all ages and sensitive subpopulations, could occur.*

lung, dogs are more similar to humans than rodents. Mechanistic studies (Li and Pauluhn, 2019) also suggest that dog models are more predictive of human phosgene toxicity than are rodent models. Thus, it may be most appropriate to base future phosgene risk assessments on data extrapolation from dogs to humans when dog data are available. Recent studies appear to focus on acquiring an additional in-depth understanding of the mechanism of action of phosgene through the use of animal models (Chen et al., 2009; Wang et al., 2013; Luo et al., 2014; Li and Pauluhn, 2017).

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Chapter 24

Carbon monoxide: can't see, can't smell, body looks red but they are dead

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24.1 Introduction

Carbon monoxide (CO) is a ubiquitous partial organic combustion product and a common cause of intentional, unintentional, and fire-related death. It is the predominant organic-derived combustion product at temperatures greater than 800°C. The breathable air concentration of CO can vary from 0.1 ppm in a clean atmosphere to 5000 ppm in the proximity of domestic wood fire chimneys (Fawcett et al., 1992). CO is a significant component of cigarette smoke (Hartridge, 1920; Hoffmann et al., 2001). Intercontinental transport of CO occurs due to its atmospheric lifetime of 1–2 months (Akimoto, 2003). Because of its ability to react with myoglobin to form red-colored carboxymyoglobin, CO has also been used to increase the visual appeal and marketability of meat products.

At toxic concentrations CO is a cause of "anemic hypoxia" in that it binds to hemoglobin with such high affinity that it prevents oxygen from binding, thus reducing the oxygen-carrying capacity of the blood. Using strict chemical weapon definitions, it is not a classical "blood agent" (i.e., it is not in the same weapon mode of action class as the cyanides, arsines, and phosgene) in that it does not prevent the exchange of oxygen and carbon dioxide between the blood and the body's cells/disrupt oxidative energy production. However, CO is often incorrectly included within the "blood agent" chemical weapon class primarily because one of its effects is to give venous blood a "cherry red" color similar to the effect of the cyanides. Notably, the causes of the venous blood color change between the cyanides and CO are different.

Although not formally listed in the Chemical Schedules of the 2005 Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and On Their Destruction, CO does have a number of potentially militarily useful features, namely (OPCW, 2005):

- It is odorless and initially non-nociceptive.
- It is initially incapacitating, produces unconsciousness, and is ultimately fatal, especially when present at high concentrations in poorly ventilated, confined spaces.
- It is heavier than air and thus tends to concentrate in low-lying spaces. Thus, it tends to displace breathable air in low-lying and/or enclosed spaces.
- It decreases the effectiveness of nonair/nonoxygen-supplied respirators and other nonair-supplied personal protective equipment since it reduces the safety of breathable air that passes through the filters of these devices.
- In enclosed environments high concentrations can be sustained over long periods of time, thus potentially exhausting and neutralizing the filters/scrubbers in personal protective equipment such as respirators.
- At high concentrations it can cause incapacitation and death relatively quickly and reputedly relatively painlessly; these properties have resulted in its use as a mass euthanasia agent for livestock (particularly poultry).
- Large quantities of the gas can be simply and easily produced, easily transported, easily and safely stored, and deployed with readily available, simple, and minimal personal protective equipment.
- Large numbers of simple improvised CO-producing devices can be rapidly produced and constructed from ubiquitous materials, for example, charcoal burners, fires, internal combustion engine exhausts, etc.

Due to these militarily attractive characteristics it has been used commonly and "informally" to both defend and

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clear bunkers, tunnel systems, and other poorly ventilated enclosed fortifications, particularly in conflicts featuring irregular/asynchronous warfare. CO will likely continue to be used "informally" in specialized military and policing situations (National Academies of Sciences, Engineering, and Medicine, 2015).

Because combustion is an inevitable consequence of warfare CO will always be present, at least to some degree, under battlefield conditions (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008; National Research Council, 2002; Institute of Medicine, 2010, 2014). In a number of modern military circumstances, combined exposure to CO and cyanide may occur. While it is likely that CO has actively or accidentally been used in warfare for centuries, the modern descriptions of its detection at concentrations of medical importance in the battlefield environment first came from reports from the western European front during World War I (Anon, 1941; Miller, 1926). Systematic programs of ethnic cleansing (Einsatzgruppen operations in Eastern Europe and Russia and the initial Holocaust-related gassing operations at Chelmno followed by the subsequent expanded gassing operations at Belzec, Sobibor, and Treblinka) and elimination of the lesser abled (the child euthanasia program and the gassing phase of the subsequent T4 action) by CO-mediated mass killing were conducted by the Nazis during World War II (Kogon et al., 1993).

The main source of CO during these operations was diesel engine exhausts generated by mobile "gas vans" or diesel engine exhausts generated at stationary gas chambers.

A modern documented example of the deliberate military defensive use of CO was by the Viet Cong who used it to inflict casualties on the "tunnel ferrets" of the Royal Australian Engineers of the Australian Army during the Vietnam War (Rotman, 2006). Acute CO poisoning also may account for at least some of the casualties associated with the use of napalm.

More recently concerns have been raised about exposures occurring within weaponized armored vehicle system crews who are exposed to the gaseous byproducts of weapons propellants, within submarine environments and within military aircraft (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008).

Nonmilitary-related, unintentional CO poisoning remains extremely common. The US Centers for Disease Control and Prevention (US CDC) has reported that over the course of the 1999–2012 period, unintentional, nonfire-related, CO poisoning caused an average of about 440 deaths per year in the United States (Jiaquan, 2014; Sircar et al., 2015). Overall this form of CO poisoning was the second most common cause of nonmedicinal poisoning deaths in the United States over this period. Death rates were about threefold higher in males compared with females and were highest amongst the elderly. Outbreaks of unintentional CO poisoning due to inappropriate (i.e., indoor, enclosed, and/or inadequately ventilated spaces) use of combustion heating devices are classically associated with periods of sudden cold weather or storms, especially when combined with energy supply and/or heating failures and/or inadequate housing and/or socioeconomic disadvantage. Such outbreaks are especially common in socioeconomically disadvantaged populations during winter storms.

Reports of mysterious illnesses, strange visions, and inexplicable deaths of entire households are most likely due to malfunctioning chimneys or other malfunctioning combustion heating devices, resulting in CO poisoning. Nonlethal, but relatively high, CO concentrations are serious threats to pregnant women and their fetuses, and individuals with preexisting cardiac diseases.

CO is also an endogenously generated gas and has an important role in normal physiology. It is also pharmacologically active. Since the central theme of this chapter is on the use of CO as a weapon, the physiological actions of CO are only cursorily discussed when appropriate to the theme of the chapter.

24.2 Historical background

The toxicity of CO was recorded by Aristotle (384–322 BCE) in the third century BCE; by the first century BCE charcoal fumes were used for suicide and executions without any knowledge of the exact nature of the killer (Lewin, 1920; Shephard, 1983). Byzantine emperor Julian the Apostate and his successor, Jovian, were poisoned in CE 363 and 364, respectively, because coal was used to heat their braziers (Lascaratos and Marketos, 1998). In 1700, Bernardino Ramazzini (1633–1714), a physician and professor at the University of Modena, recognized that fumes from burning coal by confectioners caused headache and dyspnea, and that miners encountered "noxious vapors" from burning coals that, if not vented out, could "kill a man on the instant" (Shephard, 1983; Pankow and Penny, 2000). Purified CO was first prepared by the French chemist de Lassone in 1716 who thought it was hydrogen because it burned with a blue flame. Cruikshank eventually demonstrated that the gas that burned with the blue flame was an oxide of carbon (CO) that could be converted into carbon dioxide of carbon by exploding it with oxygen.

The French physiologist Bernard described the toxicity of experimental CO in dogs in 1846. On necropsy he noted that the blood was crimson in color in all the heart chambers as well as in the veins (Bernard, 1865). Bernard, correctly, assumed that the crimson color was

due to excess of oxygen in the blood. Subsequently Haldane identified carboxyhemoglobin (COHb) and determined its chemical nature (Haldane, 1895). Haldane's first law, which remains valid, states: "When a solution containing hemoglobin is saturated with a gas mixture containing O_2 and CO the relative proportions of the hemoglobin which enters into combination with the two gases are proportional to the relative partial pressures of the two gases, allowing for the fact that the affinity of CO for hemoglobin is about three times greater than that of O_2."

Haldane subsequently discovered that CO poisoning could be treated using oxygen, which still remains the most effective antidote for CO poisoning (Haldane, 1895; Haldane and Priestley, 1935; Sluijter, 1967; Tibbles and Edelsberg, 1996). Haldane (1895) also noted that the time of onset of CO poisoning was related to allometric scaling (the time to the onset of poisoning being shorter in smaller animals that have a higher surface area to volume ratio and thus a higher respiratory minute volume per unit body mass). Based on this principle, Haldane was the first to recommend the use of mice for the detection of CO in mines. Canaries, because of their higher respiratory efficiency than mammals in combination with allometric effects, were used from 1921 to 1987 when chemical detection methods replaced their use.

24.3 Epidemiological considerations

Morbidity and mortality from CO poisoning vary from country to country because of the different standards of surveillance, differing extent of urbanization, housing conditions, source of energy, and so on. The major source of CO in the modern developed world is the burning of fossil fuels, particularly petroleum distillates and gases. Because of their higher fossil fuel combustion levels per unit land area, megacities with a population of more than 10 million have an approximately 100-fold higher level of atmospheric CO in breathable air (typically up to 10 ppm) than smaller cities and rural areas (typically 100 ppb). The breathable air levels of CO in these cities can, at times, exceed safe levels.

In most countries, CO remains a common and important cause of unintentional deaths in peacetime. However, in the United States there have been some important and recent changes in its epidemiology (Chiew and Buckley, 2014). In part these changes appear to be due to the diagnostic criteria used and the use of presumptive treatments under circumstances where CO poisoning might be a factor. In the United States the current situation is that there are large numbers of patients diagnosed with CO poisoning; however, there are relatively few deaths ($<5\%$) (Chiew and Buckley, 2014). It has been claimed that the phenomenon of high case rates combined with low death rates is mostly due to: (1) malfunctioning or emergency improvised domestic heating (compared with fires or suicide in other locations); and (2) CO poisoning is often presumptively diagnosed and treated with supplemental oxygen at the site of assumed exposure (typically situations where smoke inhalation may have occurred) even in the absence of elevated blood COHb (Chiew and Buckley, 2014). In the United States it is rare for hospitalized patients with proven CO poisoning to have blood COHb over 50% (Chiew and Buckley, 2014). This may imply that individuals with more severe forms of poisoning are, more often than not, simply found dead.

The situation outside the United States remains quite different. In many countries severe and/or fatal CO poisoning (intentional or otherwise) remains common. In Australia and several other Asian countries deaths due to suicidal CO poisoning remain very common, with deaths often outnumbering the number of patients admitted for treatment (Chiew and Buckley, 2014). Many of these deaths are associated with the use of internal combustion engine exhaust or charcoal burners. It has been claimed that CO-associated suicide epidemics have occurred in some countries following media claims that such deaths are painless and free from violence (Chiew and Buckley, 2014). Of note are findings from the United States and Europe that the installation of catalytic converters into motor vehicles has reduced the completion of suicide rate and CO poisoning rates associated with vehicle exhausts. The cessation of use of coal gas for domestic heating and cooking gas supply also had a similar effect in the United Kingdom (Surtees and Duffy, 1989).

24.4 Physicochemical properties of carbon monoxide

CO is a colorless, odorless, tasteless, noncorrosive, stable, nonradical, diatomic, atmospheric molecule. CO has low water solubility and negligible quantities dissolve in blood at normally encountered pressure (Shephard, 1983). CO has a shorter interatomic distance ($\sim$1.13 Å) than would be anticipated for a single bond. It has high heat formation from the constituent atoms (bond strength 2.07 MJ/mol) and the electric charge is distributed rather uniformly between the carbon and oxygen atoms. Pauling (1960) postulated that under normal circumstances CO existed as a hybrid containing approximately equal proportions of three distinct chemical structures. The general physicochemical properties of CO are presented in Table 24.1.

24.5 Sources of carbon monoxide

There are two main sources of CO—exogenous and endogenous. Although atmospheric CO is the principal

TABLE 24.1 Physicochemical properties of carbon monoxide.

Property	Description
Chemical structure	:C≡O:
Molecular weight	28.01
Critical point	−140°C at 3495 kPa
Melting point	−199°C
Boiling point	−191.5°C
Fundamental vibration transition	2143.3 cm^{-1} (4.67 μm)
Density at 25°C, 101.3 kPa	1.145 g/L
Specific gravity relative to air	0.967
Solubility in water at 0°C	35.4 mL/L
Solubility in water at 25°C	21.4 mL/L
Explosive limit in air	12.5%–74.2%

cause of CO toxicity, endogenous produced CO can, under certain conditions, become pathological (Marks et al., 1991; Maines, 1997; Nezhat et al., 1996; Wu and Wang, 2005).

24.5.1 External sources of carbon monoxide

CO is always a product of incomplete combustion of organic materials, including fossil fuels. It is commonly encountered in the operation of vehicles, heating, coal power generation, and biomass burning (Godish, 2003). Leaking motor vehicle exhausts, operation of motor vehicles in enclosed spaces and driving vehicles with the tailgate open, and wakeboarding behind boats are important sources of exposure.

Natural geographical events such as volcanic eruptions, emission of natural gases, degradation of materials of biological origin, and forest fires all contribute to atmospheric CO. Approximately 40% of global CO comes from these natural sources. Anthropogenic activities such as fossil fuel consumption, garbage disposal, tobacco smoke, and charcoal fires contribute to the remaining 60% of global CO production (Jain, 1990; Vreman et al., 2000). Because human activity and density differ from place to place, atmospheric CO levels also vary greatly from place to place. In the United States CO emissions, and air levels, underwent a rapid decline from 1970 to 2010 and have continued to slowly decline since that time. Apart from various other changes, the developing world has been characterized by increasing migration of rural populations to slums and shanty towns on the outskirts of cities. This phenomenon has been associated with an increase in CO levels in city atmospheres which can, on occasion, exceed safe levels in breathable air.

24.5.2 Endogenous sources of carbon monoxide

The major source of endogenous CO in a healthy individual is from the degradation of heme by heme oxygenases (HO-1 is inducible and HO-2 is constitutive). Heme oxygenases degrade heme into CO and biliverdin, with the latter being rapidly converted into bilirubin (Coburn et al., 1963, 1967; Coburn, 1979; Mores and Sethi, 2002). This process is the rate-limiting step in the endogenous production of CO and accounts for about 86% of endogenous CO production. Notably, the older an individual erythrocyte, the greater is its heme catabolism and CO output. In neonates, red blood cells have a shorter lifespan, producing two- to three-times more endogenous CO than adult erythrocytes (Fallstrom, 1968). Fetal hemoglobin also has a higher affinity for CO binding. The rate of endogenous CO production and excretion also parallels the rate of bilirubin production. Thus, blood COHb levels can be used as an indirect measure of heme degradation and bilirubin production. A measurement of end-tidal CO in breath corrected for inhaled CO is also used as a measure of assessing infants at risk for severe hyperbilirubinemia because CO and bilirubin are produced in equimolar amounts (Bartoletti et al., 1979). The remainder of endogenous CO production derives from other hemoproteins such as myoglobin and many other iron-containing enzymes (Coburn, 1970; Vreman et al., 2000). Various medical procedures can also generate CO and increase blood COHb (Baum et al., 1995; Fang et al., 1995; Moon et al., 1992; Nezhat et al., 1996; Vreman et al., 2000; Wu et al., 1998).

24.6 Methods for carbon monoxide measurement

Appropriate techniques exist for the quantitation of CO in gaseous samples and tissue samples. In either case, caution needs to be exercised to ensure that CO concentration is not altered by interaction with the sample vessel during storage and transport. For example, Vacutainer tubes were found to greatly alter CO levels when used for storage of blood samples (Vreman et al., 1984). With the recognition of the hazardous nature of CO and its almost universal presence, several sophisticated methods for quantifying CO have been developed (IPCS, 1979; Vreman et al., 2000). Both gas chromatography and spectrophotometry are considered appropriate, although the former is favored (Coburn et al., 1964; Collison et al., 1968; Vreman et al., 1984, 1998; Constantino et al., 1986). The development and ready

availability of many forms of real-time personal and space CO monitors and alarms (including devices for household use) is a relatively recent and welcome development. These devices have no doubt prevented many cases of CO poisoning and have saved many lives.

24.7 Measurement of blood carbon monoxide

Blood CO is still mostly determined by measuring blood COHb levels because of the very high (200–240 times that of oxygen) affinity for CO for hemoglobin (Piantadosi, 1999). Notably COHb levels from venous blood samples appear to correlate with those in arterial blood samples. Fetal blood COHb levels are typically about 30% higher than maternal blood levels, because fetal hemoglobin has a higher affinity for CO than adult hemoglobin.

COHb in blood is quite stable and its concentration does not change over a long period (up to 6 months) provided the sample is stored in the dark and under sterile conditions. Blood levels of COHb are not normally expected to exceed 5% at ambient levels of CO. Several techniques for measuring COHb exist (IPCS, 1979). The more sensitive techniques require the release of CO from hemoglobin into a gas phase followed by direct detection by infrared absorption, difference in thermal conductivity between CO and the carrier gas, amount of ionization after conversion of CO to methane, or the release of mercury vapor resulting from interaction of CO with mercuric oxide. The conventional method of expressing CO in blood samples is as percent COHb, which is calculated using the following formula:

$$\%\ \text{COHb} = \frac{\text{Blood CO concentration mL/100mL of blood}}{(\text{Hb g/100mL of blood} \times 1.389)} \times 100$$

The factor of 1.389 is the combining capacity of CO for hemoglobin in mL of CO/g of hemoglobin. Most modern clinical pathology laboratories use dedicated blood co-oximeters (a form of dedicated spectrophotometer), which simultaneously measure total hemoglobin and fractional (%) values for COHb, oxyhemoglobin, deoxyhemoglobin, and methemoglobin, thus negating the need for manual calculations. Critically, determination of peripheral blood oxygen saturation with typical simple two-wavelength pulse oximeters will not take into account the presence of COHb in the blood and will potentially give spuriously high indications of tissue oxygenation.

24.8 Ambient air carbon monoxide

Because CO concentrations in ambient air and at workplaces are usually quite low, reliable methods for sample collection and transport, and also highly sensitive methods for measurement, are needed (Smith and Nelson, 1973; IPCS, 1979). The sampling method recommended by WHO (IPCS, 1979) comprises a sample introduction system consisting of a sampling probe, an intake manifold, tubing, and air remover. Known gas concentrations are periodically collected to verify the method. According to IPCS (1979), the analyzer system consists of an analyzer as well as sample preconditioning components fitted with a moisture-control system such as the nondispersive infrared (NDIR) analyzer. The infrared absorption near 4.6 μm, characteristic of CO, is used to measure its concentration. The most sensitive analyzers can detect CO concentrations as low as 0.05 mg/m^3 (0.044 ppm). The NDIR analyzer designed by Luft (1962) is considered appropriate because it is little affected by flow rate, requires no wet chemicals, has a short response time, and is sensitive over wide concentration ranges.

Other analyzers include gas chromatography, which is a sensitive, automated, and semicontinuous technique in which CO is separated from water, CO_2, and hydrocarbons (other than methane) by a stripper column and CO is passed through a catalytic reduction tube where it is converted to methane. The converted CO is passed through a flame ionization detector; its sensitivity range is 0.026–43.7 ppm (IPCS, 1979). Other methods such as small personal exposure monitors can measure CO concentrations on a continuous basis and store data on internal digital memories (Ott et al., 1986).

24.9 Home detectors

Residential CO detectors are designed like smoke detectors and provide protection from excessive CO concentrations inside homes by sounding alarms. They are based on an interactive-type sensor, such as tin oxide or artificial Hb that relies on an interaction between CO, and the sensitive element to generate an alarm. The alternate current-powered home detectors have a metallic sensor that reacts with CO. The battery-powered ones have a chemically treated gel that darkens on exposure to CO. They are all designed to sound an alarm within 90 min at CO concentrations of 100 ppm, within 35 min at 200 ppm, or within 15 min at 400 ppm (IPCS, 1979). Inhalation CO concentration of 400 ppm can relatively increase COHb to 10%, with toxicity occurring at levels higher than 10%.

24.10 Carbon monoxide in expired breath

Measurement of CO in the expired breath is based on the assumption that CO in alveolar air is in equilibrium with the partial pressure of CO in blood, which, in turn, is in equilibrium with CO bound to hemoglobin that is COHb (Douglas et al., 1912).

24.11 Toxicokinetics and toxicodynamics

24.11.1 Absorption, distribution, and elimination of carbon monoxide

Although the biological effects of CO differ depending on whether it is inhaled or endogenously produced, the ultimate fate of CO is the same regardless of its source. Exogenous CO reaches the body solely by pulmonary absorption. Once inhaled, CO combines reversibly with hemoglobin and, to a smaller extent, with myoglobin and other iron-containing macromolecules. When reacting with ferrous iron, carbon atoms of CO form a sigma bond, also involving a D-orbital electron from the third shell of Fe^{2+} (Shephard, 1983). In the body, Fe^{2+} also has four nitrogen linkages that contribute a substantial electron density. The effective valency is less than Fe^{2+}, enabling a more readily reversible reaction with both oxygen and CO. The solubility of CO in water is approximately 20% less than that of O_2 and for practical toxicological purposes absorbed CO is effectively absent from the plasma.

Because of the very high CO-binding capacity of hemoglobin within the blood, any CO diffusing into the blood becomes rapidly bound and thus does not contribute to the blood partial pressure of CO. This results in the maintenance of a large partial pressure gradient across the pulmonary capillary bed irrespective of pulmonary blood flow (i.e., right cardiac output) and alveolar ventilation rate (thus it is neither perfusion nor ventilation limited). The limiting factor for absorption of inhaled CO is its ability to diffuse across alveolar membranes. Thus, the inhaled absorption of CO is regarded as being "diffusion limited," a physiological property that is almost unique to CO. Because inhaled CO's partial pressure in the pulmonary capillary blood remains negligible across the entire alveolar membrane it is especially useful for measuring the diffusing capacity of the lung.

Because most of the CO in the body is bound to hemoglobin, the relative affinities of CO and oxygen for hemoglobin are of critical significance in terms of both its toxicity and excretion. This relationship was first described by Haldane (1922; Haldane's first law):

$$\frac{\text{COHb}}{\text{HbO}_2} = M \times \frac{p_{\text{CO}}}{p_{\text{O}_2}}$$

M is the relative affinity of hemoglobin for CO compared with oxygen (between 208 and 245 at 37°C). The Haldane relationship between CO, oxygen, and hemoglobin explains both the high toxic potential of CO and the advantages of hyperbaric oxygen treatment of CO poisoning (Pace et al., 1950; Peterson and Stewart, 1970; Jay and McKindley, 1997). The blood level of COHb as a percent of total hemoglobin is directly related to the exposure duration at any concentration of CO (Fig. 24.1; derived from the Coburn, Forster, and Kane equation). Table 24.2 shows the equilibrium percentage saturation of hemoglobin with CO at various alveolar pressures of CO, calculated using Haldane's first law (IPCS, 1979). Based on human experimental data there are several empirical models for predicting blood COHb levels as a function of ambient CO concentration and exposure time (IPCS, 1979). Under most circumstances the best all-around model for blood COHb prediction under military circumstances is the Coburn, Forster, and Kane equation (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008; IPCS, 1979):

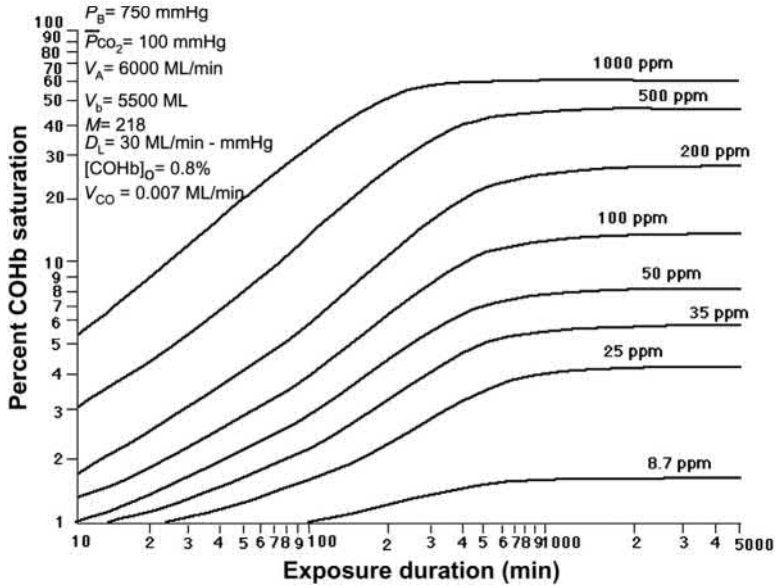

FIGURE 24.1 COHb for different exposure concentration−time combinations as predicted by the Coburn, Forster, and Kane equation. *From Chapter 2, Carbon Monoxide. National Research Council. 2010. Acute Exposure Guideline Levels for Selected Airborne Chemicals, vol. 8. The National Academies Press, Washington, DC. doi: 10.17226/12770.*

TABLE 24.2 The equilibrium percentage saturation of hemoglobin with carbon monoxide at various alveolar pressures of carbon monoxide, calculated using Haldane's first law (IPCS, 1979).[a]

% HbCO	mg/m^3	ppm	% in air	Pa	Torr
0.87	5.7	5	0.0005	0.506	0.0038
1.73	11.5	10	0.001	1.013	0.0076
3.45	23.0	20	0.002	2.026	0.0152
5.05	34.5	30	0.003	3.305	0.0248
6.63	46.0	40	0.004	4.052	0.0304
8.16	57.5	50	0.005	5.065	0.0380
9.63	69.0	60	0.006	6.078	0.0456
11.08	80	70	0.007	7.091	0.0532
12.46	92.0	80	0.008	8.104	0.0608
13.80	103.0	90	0.009	9.117	0.0684
15.11	114.5	100	0.010	10.130	0.0760
16.37	126.0	110	0.011	11.143	0.0836
17.60	130.0	120	0.012	12.156	0.0912
18.78	149.0	130	0.013	13.170	0.0988
19.95	160.0	140	0.014	14.183	0.1064
21.05	172.0	150	0.015	15.196	0.1140
22.15	183.0	160	0.016	16.209	0.1216
23.23	195.0	170	0.017	17.209	0.1291
24.26	206.0	180	0.018	18.235	0.1368
25.25	218.0	190	0.019	19.221	0.1442
26.22	229.0	200	0.020	20.261	0.1520

[a]*The alveolar oxygen pressure is assumed to be 13 kPA (98 Torr).*

$$\frac{(A \times \mathrm{HbCO}_t - (\mathrm{BV}_{\mathrm{CO}} + \mathrm{PI}_{\mathrm{CO}}))}{(A \times \mathrm{HbCO}_0 - (\mathrm{BV}_{\mathrm{CO}} + \mathrm{PI}_{\mathrm{CO}}))} = e^{-tAV_bB}$$

where:

$A = P_{c,O2}/M$ [HbO_2] where:
$P_{c,O2}$ is the average partial pressure of O_2 in lung capillaries (approximately 110 mmHg at sea level)
M is the ratio of the affinity of blood for CO to that for O_2 (approximately 218)
HbO_2 is the mL of O_2/mL blood, or $= 0.22 - [\mathrm{HbCO}]_t$
$[\mathrm{HbCO}]_t$, is mL of CO/mL blood at time t, or $= [\mathrm{COHb\%}]_t \times 0.0022$
$B = 1/DL_{CO} + PL\ V_A$ where:
DL_{CO} is diffusivity of the lung for CO (mL/min/mmHg), or $= 35VO_2.\ e^{0.33}$
V_A is alveolar ventilation rate (mL/min), or $= 0.933\ V_E - 132f$ and V_E is the ventilation volume (mL/min) and f is the ventilation frequency
PL is barometric pressure minus the vapor pressure of water (i.e., 49) at body temperature (mmHg)
V_{CO} is the rate of endogenous CO production (mL/min); approximately 0.007 mL/min
PI_{CO} is the is the average partial pressure of O_2 in lung capillaries (at sea level 110 mmHg)
$[\mathrm{HbCO}]_0$ is the mL of CO/mL blood at the beginning of the exposure (approximately 0.8% COHb, or 0.0176 mL CO/mL blood for a nonsmoker)
e is the base of natural logarithms (2.7182)
t is the exposure time (minutes)
V_b is the blood volume (mL) (typically assumed to be 74 mL/kg body weight)

Table 24.3 shows the predicted blood HbCO for males with various levels of activity and various breathable air CO concentrations based on the Coburn, Forster, and Kane equation (IPCS, 1979).

TABLE 24.3 Predicted blood COHb for males with various levels of activity and various breathable air CO concentrations.

Time	200 ppm			100 ppm			75 ppm			50 ppm		
	S	L	H	S	L	H	S	L	H	S	L	H
15 min	1.8	3.5	5.2	1.2	2.0	2.8	1.0	1.6	2.2	0.82	1.2	1.6
30 min	3.1	6.2	9.2	1.8	3.3	4.8	1.5	2.6	3.7	1.1	1.9	2.6
45 min	4.3	8.7	12.6	2.4	4.6	6.5	1.9	3.5	4.9	1.4	2.5	3.4
60 min	5.5	11.0	15.5	3.0	5.7	7.9	2.3	4.3	6.0	1.7	3.0	4.1
90 min	7.7	14.9	20.2	4.0	7.6	10.2	3.1	5.8	7.7	2.2	4.0	5.2
2 h	9.7	18.1	23.7	5.0	9.2	11.9	3.9	7.0	9.0	2.7	4.7	6.1
4 h	16.3	26.2	30.4	8.3	13.2	16.3	6.3	10.0	11.5	4.4	6.9	7.7
6 h	21.1	30.0	32.4	10.7	15.1	16.2	8.1	11.3	12.2	5.5	7.6	8.2
8 h	24.5	31.7	32.9	12.4	15.9	16.5	9.4	12.0	12.4	6.4	8.0	8.3
24 h	32.7	33.2	33.2	16.5	16.7	16.6	12.4	12.5	12.5	8.4	8.4	8.3
Infinity	33.4	33.2	33.2	16.8	16.7	16.6	12.7	12.5	12.5	8.5	8.4	8.3
Time	**35 ppm**			**25 ppm**			**10 ppm**			**5 ppm**		
	S	L	H	S	L	H	S	L	H	S	L	H
15 min	0.72	1.0	1.3	0.66	0.84	1.0	0.55	0.61	0.67	0.52	0.54	0.56
30 min	0.93	1.4	1.9	0.80	1.2	1.5	0.61	0.72	0.82	0.54	0.57	0.60
45 min	1.1	1.9	2.5	0.95	1.4	1.9	0.66	0.81	0.95	0.56	0.61	0.64
60 min	1.3	2.2	3.0	1.1	1.7	2.2	0.71	0.90	1.1	0.58	0.63	0.68
90 min	1.7	2.9	3.7	1.3	2.1	2.7	0.80	1.1	1.2	0.62	0.69	0.74
2 h	2.0	3.4	4.3	1.6	2.5	3.1	0.89	1.2	1.4	0.66	0.73	0.78
4 h	3.2	4.7	5.4	2.4	3.4	3.9	1.2	1.5	1.6	0.77	0.84	0.86
6 h	4.0	5.4	5.7	2.9	3.9	4.1	1.4	1.6	1.7	0.85	0.88	0.88
8 h	4.5	5.7	5.8	3.3	4.1	4.2	1.5	1.7	1.7	0.91	0.91	0.89
24 h	5.9	5.9	5.9	4.3	4.2	4.2	1.9	1.8	1.7	1.05	0.93	0.89
Infinity	6.0	5.9	5.9	4.4	4.2	4.2	1.9	1.8	1.7	1.06	0.93	0.89

H, Heavy physical work; *L*, light physical work; *S*, sedentary subjects.

Table 24.4 shows the exposure conditions that would prevent COHb levels exceeding 5% in nonsmoking occupational groups performing light and heavy physical work (IPCS, 1979).

Under normal physiological conditions there is almost always a certain amount of COHb even when breathing CO-free air because the breakdown of Hb results in endogenous production of CO. This can result in 0.5%–0.8% COHb in normal blood (Lawther, 1975). At any CO concentration in the air COHb will attain an equilibrium and if CO in the air is lower than that required for a given COHb % at equilibrium, then CO will leave COHb and be exhaled until a new equilibrium is established (Fig. 24.1). This has practical implications. For example, a smoker with a relatively high COHb at equilibrium may exhale CO. Also, if the basal COHb is high, then an equilibrium at a high CO concentration in the air will be achieved more quickly than it would be at an initial low COHb.

In practical toxicological terms, CO is eliminated from the body by exhalation. The rate of excretion is primarily dependent on the amounts of CO and oxygen present, the magnitude of ventilation, and the quality of the alveolar diffusion barrier (IPCS, 1979). As a general rule the alveolar diffusion barrier thickens with age, potentially resulting in slower rates of excretion. There are mixed data on

TABLE 24.4 Exposure conditions that would prevent blood COHb levels exceeding 5% in nonsmoking occupational groups performing light and heavy physical work.

Concentration		Exposure time not to be exceeded		Concentrations that would produce 5% HbCO		Safety factor	
ppm	mg/m^3	Light work	Heavy work	Light work	Heavy work	Light work	Heavy work
200	230	15 min	–	298	–	1.5	–
100	115	30 min	15 min	157	193	1.6	1.9
75	86	60 min	30 min	87	105	1.2	1.4
50	55	90 min	60 min	64	62	1.3	1.2
35	40	4 h	2 h	37	41	1.1	1.2
25	29	8 h	8 h	31	30	1.2	1.2

whether or not biological sex affects excretion rates. The CO blood elimination curve is consistent with a two-compartment elimination model with an initial sharp decline in blood levels over the first 20 min following the cessation of exposure. This "distribution phase" is associated mostly with redistribution to splenic blood, myoglobin, and cytochrome enzymes. This is followed by a slower "elimination phase," which reflects the release of CO from hemoglobin and myoglobin, pulmonary diffusion, ventilation, as well as the fact that pCO generally decreases over time. The whole-body elimination phase kinetics is nonfirst order, that is, the elimination half-life is inversely proportional to blood COHb and is also affected by inhaled oxygen concentration. Under commonly encountered conditions the whole-body elimination half-life ranges from 2 to 6.5 h. This means that high blood COHb levels can occur even at relatively low levels of CO in the inspired air. Considerable interindividual variation in CO elimination is encountered. Previous sustained CO exposure may slow elimination (IPCS, 1979). The elimination half-life of CO in a healthy adult can be reduced by at least a factor of fourfold by the use of supplemental oxygen (Roughton and Root, 1945; Pace et al., 1950; Bartlett, 1968).

As is predictable from the Haldane equation, elimination is much slower under conditions of lower atmospheric oxygen partial pressure. This has been confirmed by studies of human subjects at high altitude (IPCS, 1979). Conversely, increasing the content and/or partial pressure of oxygen in inspired air results in an increased rate of excretion. This is the basis of the currently most reliable antidote for CO poisoning, that is, oxygen at normal or hyperbaric pressures. At high blood COHb levels (40%–70%) a 1-h exposure to a hyperbaric oxygen-enriched environment [oxygen level equivalent to 2.5 atmospheres, partial pressure of oxygen equal to 253 kPa (1900 Torr) or an alveolar oxygen pressure of 239 kPa (1801 Torr)] resulted in a reduction of blood COHb level to about 10%–15% of the initial level (IPCS, 1979). Based on animal data and under conditions of high blood COHb levels (60%–74%), 50% of the absorbed CO was excreted in 19 min when 5% carbon dioxide and 95% oxygen at normal pressures were breathed, compared with a time of 28 min for 100% oxygen, and 41 min for ambient air.

24.12 Mechanism of toxicity

24.12.1 Classical mode of action

The classical toxicological mode of action of CO is tissue hypoxia (Chiew and Buckley, 2014) due to several key events: (1) the preferential binding of CO to hemoglobin due to its >200-fold higher affinity for this molecule than oxygen; (2) the uptake of CO by Hb is very rapid, however the release of CO from COHb complex is slow, resulting in relatively slow excretion via exhalation; (3) as the concentration of COHb increases, the formation of oxyhemoglobin at any concentration of oxygen in the inhaled air decreases; (4) COHb cannot carry oxygen, reducing the oxygen-carrying capacity of the blood; and (5) COHb increases the affinity of the remaining hemoglobin oxygen-binding sites, shifting the oxygen–hemoglobin dissociation curve to the left. The sum total of these effects triggers homeostatic increased cardiac output and increased respiratory rate in order to maintain brain oxygenation and carbon dioxide excretion. Unfortunately, this homeostatic response has the overall effect of increasing CO uptake, exacerbating the pathophysiological situation.

Once a critical level of COHb is reached (which is individually variable) the homeostatic responses are

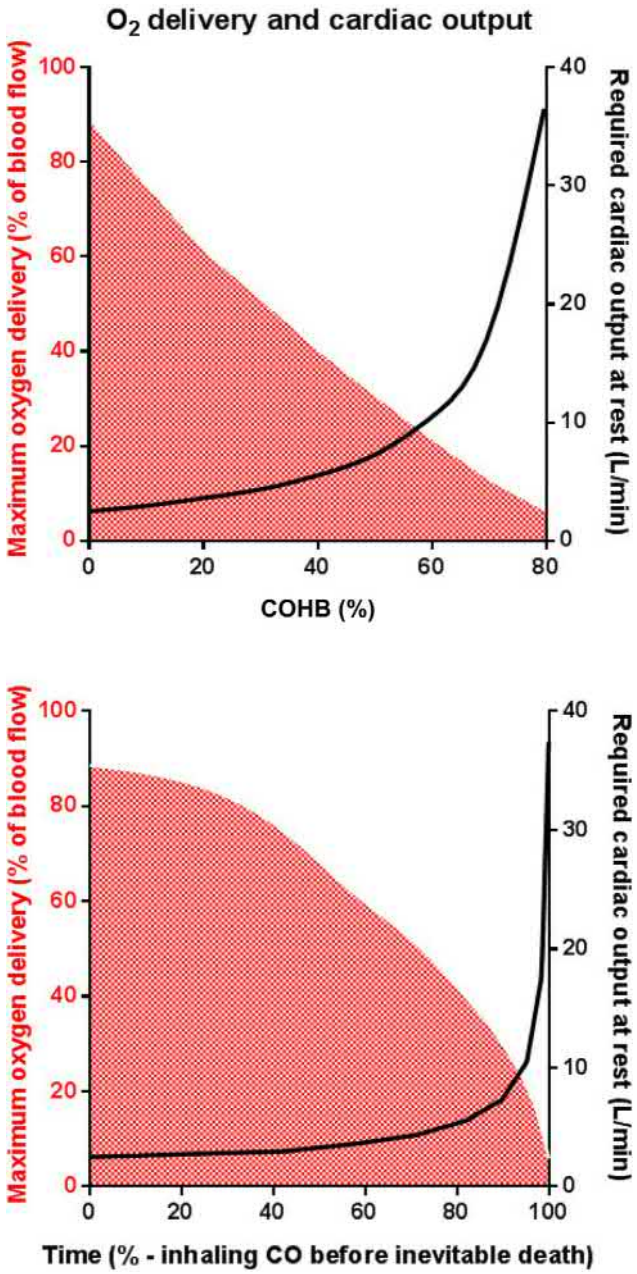

FIGURE 24.2 Carboxyhemoglobin (COHb) versus oxygen delivery capacity of the blood and increased cardiac output required to deliver the same amount of oxygen. (A) This is shown with a linear progression in COHb. (B) In the more likely scenario, there is continuous exposure to COHb and the rate of uptake of COHb is roughly proportional to cardiac output, resulting in a very rapid deterioration to life-threatening poisoning, after a relatively long mild poisoning stage. CO, carbon monoxide; O_2, oxygen. *Reproduced from Chiew, A., Buckley, N., 2014. Carbon monoxide poisoning in the 21st century. Crit. Care 18:221–229.*

unable to compensate for the decreased blood oxygen-carrying capacity and tissue oxygen availability, cardiac hypoxia results in decreased cardiac output exacerbating tissue hypoxia and then death ensues. These relationships are shown in Fig. 24.2 (Chiew and Buckley, 2014). Notably some of the early nonspecific, clinical signs and symptoms of CO poisoning (headache, nausea, and tachycardia) that occur at blood COHb of less than about 40% are more related to the compensatory homeostatic response rather than overt tissue hypoxia.

The biomarkers of the serious toxicity that occurs following the blood COHb level reaching the pathophysiological "tipping point," are sustained unconsciousness and evidence of brain hypoxia (Glasgow coma score of less than 9, seizures), increased blood cardiac injury biomarkers (cardiac troponin I level greater than or equal to 0.7 ng/mL, or creatine kinase-MB level greater than or equal to 5.0 ng/mL, and/or diagnostic electrocardiogram changes), increased serum S100B protein (greater than 0.165 μg/L), systolic blood pressure <90 mmHg, elevated creatine phosphokinase concentration, and leukocytosis (Pepe et al., 2011; Park et al., 2012; Henry et al., 2006). These findings are correlated with poor clinical outcomes.

24.12.2 Electrocardiographic/heart rhythm effects

Both intermittent and continuous CO exposure are arrhythmogenic in animals (Raub and Benignus, 2002). These effects have been presumed to be due to cardiac ischemia and/or sensitization of the myocardium. Indirect effects associated with changes in platelet aggregability have also been proposed. Exercise exacerbated the effects in some studies.

24.12.3 Cardiac hemodynamic effects

Although there are contradictory data in animal studies there is a general consensus that CO in sufficiently high doses can deleteriously affect many cardiac hemodynamic variables (Raub and Benignus, 2002). Homeostatic increases in coronary blood flow in response to increasing blood COHb levels have been demonstrated. Limited data suggest that once blood COHb levels of 6%–7% are achieved there is no further increase in coronary blood flow. This suggests that even small increases in blood COHb may be problematic in cardiac-disabled individuals. Relative underperfusion of the myocardial subendocardial layer (particularly of the left ventricle) and lowered local PO_2 have been demonstrated in animal models at blood COHb levels of 41%.

24.12.4 Cardiomegaly

Subchronic repeated daily exposure to moderate levels of CO has been repeatedly demonstrated to cause reversible whole heart cardiomegaly in animal models (Raub and Benignus, 2002). The cardiomegaly was accompanied by changes in cardiac lactate dehydrogenase isoenzyme composition that were similar to those reported in other

conditions that cause cardiac hypertrophy. In rats, the exposure threshold for the effect is about 200 ppm of CO in air, equating to about 12% blood COHb.

24.12.5 Other cardiac effects

The interaction of CO with myoglobin can also explain some of its toxic effects. This effect impairs oxygen supply to cardiac muscle mitochondria, resulting in an effect similar to myocardial ischemia. Patients with underlying cardiac conditions are therefore at high risk for cardiac arrhythmias after exposure to CO (DeBias et al., 1976; Henz and Maeder, 2005; Olson, 1984; Sangalli and Bidanset, 1990) as well as chest pain and death. The interaction of CO with skeletal muscle myoglobin can also cause muscle weakness (Herman, 1998; Wolf, 1994; Richardson et al., 2002).

24.12.6 Effects on cerebral blood flow

CO exposure triggers the stereotypical homeostatic response of the cerebral vasculature to hypoxia, namely increasing cerebral blood flow in order to maintain cerebral oxygen delivery and/or by increasing oxygen extraction in order to maintain cerebral oxygen utilization (Raub and Benignus, 2002). Notably, global cerebrovascular resistance decreases more with CO exposure than simple hypoxia and there are brain regional differences in tissue injury associated with severe CO poisoning. Brain regions that appear to be particularly sensitive are those with normally high blood flow and oxygenation requirements, such as the caudate nucleus and midbrain. The blood supply to the neurohypophysis, because of its unique physiological regulation in relation to hypoxia, does not change even with relatively severe CO poisoning.

24.13 Effects on brain metabolism

CO binds to many heme-containing proteins such as myoglobin, guanylyl cyclase, and cytochrome oxidase (Chance et al., 1970; Hill, 1994; Omaye, 2002; Kao and Nanagas, 2006). This results in an inhibition of cellular metabolism that remains even after COHb levels decline to within the normal range (Olson, 1984; Brown and Piantadosi, 1992; Piantadosi et al., 1995). However, the affinity of CO for cytochrome oxidase is very low and this interaction typically requires very high (lethal) CO concentrations (Prockop and Chichkova, 2007).

24.14 Redox and reoxygenation/reperfusion injuries in the brain

Even at sublethal concentrations, binding of CO to cytochrome may lead to the generation of superoxides (Zhang and Piantadosi, 1992; Hardy and Thom, 1994) and interference with cellular respiration. Inflammatory changes in acute CO poisoning also include neutrophil activation following interactions with platelets resulting in degranulation and perivascular oxidative stress. Additionally, CO promotes neutrophil adhesion to the microvasculature, resulting in the activation of xanthine oxidase and generation of oxidative radicals. Nitric oxide (NO) is also claimed to be an important mediator of CO-poisoning-induced oxidative effects (Kao and Nanagas, 2006). The overall end result of these effects is brain lipid peroxidation, one of the likely causes of CO poisoning-associated delayed neurological sequelae (along with changes in cerebral blood flow; Thom, 1990, 1993; Hardy and Thom, 1994; Thom et al., 1994, 1997, 2001; Ischiropoulos et al., 1996; Gilmer et al., 2002; Zhang and Piantadosi, 1992). Unfortunately, many of the studies that support these claimed modes of action lack positive controls, thus it is unclear if the observed effects are CO-specific or just oxidative effects associated with stereotypical tissue reperfusion injury.

24.15 The catecholamine crisis hypothesis

The basis for this hypothesis is that CO and sympathomimetics, such as cocaine and the amphetamines, produce similar characteristic basal ganglia injuries. According dopamine excess in the presence of hypoxia may result in neuronal excitotoxicity in the brain dopaminergic and/or serotonergic regions. This may explain the initial (acute) phase of CO-associated neurotoxicity. The hypothesis further posits that dopamine and/or serotonin excess can sustain for several weeks in the synapses in deep white matter, enhancing the oxidative metabolism of dopamine-generating reactive species and triggering abnormal inflammatory responses. As a result, serotonergic axonal injury and secondary myelin damage may lead to the delayed leukoencephalopathy or CO-associated DNS, in which leukoencephalopathy can be found in the white matter. A similar hypothesis has been proposed for 3,4-methylenedioxy-methamphetamine (MDMA) leukoencephalopathy.

24.16 Other possible mechanisms of central nervous system toxicity

Other potentially important events in CO-induced central nervous system damage include: (1) CO activation of guanylyl cyclase which triggers cerebrovascular vasodilation and cerebrovascular hypotension (Verma et al., 1993; Snyder and Ferris, 2000; Wu and Wang, 2005); (2) immune-mediated effects secondary to oxidative

damage to myelin basic protein (Ernst and Zibrak, 1998; Prockop and Chichkova, 2007; Thom et al., 2004); (3) glutamate-induced neuronal excitotoxicity (Ishimaru et al., 1992; Penny and Chen, 1996; Piantadosi et al., 1997); (4) atherogenesis (Lightfoot, 1972; Thom et al., 1999); and (5) apoptosis (Piantadosi et al., 1997).

24.17 Toxicity of carbon monoxide

24.17.1 Factors affecting susceptibility to poisoning

Important factors affecting individual susceptibility to CO poisoning are generally those that either produce higher initial COHb levels or affect the capacity of an individual to compensate for the decreased blood oxygen-carrying capacity. Common factors that fit into these categories include:

- Tobacco smoking due to increased initial blood COHb and effects on the cardiovascular and respiratory systems;
- Concurrent heart disease;
- Anemia;
- Concurrent exposure to agents that bind to hemoglobin and produce hemolysis and/or anemia, for example, dinitrotoluenes, nitroanilides, arsine, PAPP, other blood agent chemical weapons;
- Concurrent exposure to agents that produce methemoglobinemia, for example, nitrites;
- Respiratory diseases including asthma and chronic obstructive pulmonary disease;
- Lung damage due to inhalation of superheated gasses/air during following fire exposure;
- Concurrent physical exertion;
- Concurrent exposure to an oxygen-deprived environment;
- Concurrent exposure to high levels of both oxidative and/or acidic and/or particulate air pollution;
- Concurrent cyanide poisoning: some degree of concurrent cyanide poisoning is reputed to be common following some types of smoke inhalation. Concurrent cyanide exposure may also be relatively common in the battlefield environment;
- Concurrent exposure to hydrogen sulfide. A potentially common occurrence in enclosed environments containing decaying organic material, during coal mining, during petroleum and petroleum gas extraction, etc.;
- Concurrent exposure to agents that reduce central respiratory drive, for example, alcohol, barbiturates, promazine drugs, opioids, etc.;
- Concurrent exposure to agents affecting the cardiovascular system, for example, beta-blockers, calcium-channel blockers, nitrates, etc.;
- Concurrent hypothermia;
- Fetal stage of development due to the higher CO-binding affinity of fetal hemoglobin;
- Infants due to the presence of fetal hemoglobin;
- Pregnancy, particularly late-stage pregnancy, due to increased basal cardiovascular and respiratory system demand (and other factors);
- High altitude;
- Any factor that shifts the hemoglobin−oxygen dissociation curve to the left [decreased body temperature, decreased 2,3-diphosphoglycerate, decreased blood (H^+)];
- Concurrent exposure to noise exacerbates acute CO poisoning-associated hearing loss.

Other factors often relate to allometric parameters (e.g., higher respiratory minute volumes per unit body mass). These include:

- Fetal stage of development due to allometric factors;
- Infants due to allometric factors;
- Smaller body size in animals due to allometric factors, for example, mice are likely to die more quickly than humans due to their higher respiratory minute volume per unit of body mass;
- Respiratory efficiency, for example, the respiratory efficiency of birds on a per unit body weight basis is higher than that of mammals;
- Reduced capacity to escape, for example, alcohol consumption or use of other debilitating psychotropics, physically trapped, etc.

24.17.2 Combined exposures to carbon monoxide, cyanides, and other toxicological gases in battlefield and military circumstances

Modern weapons emissions are known to include CO, hydrogen cyanide, and other gases (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008). Furthermore, in naval operations combined exposures to CO, cyanides, gaseous ammonia, chlorine, vaporized hydrogen chloride, hydrogen sulfide, nitrogen dioxide, and sulfur dioxide may occur (National Research Council, 2002). The United States Army has, based on weight of evidence, concluded that (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008):

> *the adverse effects of CO and HCN at lethal and incapacitating concentrations inhaled over periods of about 30 minutes or less are additive. However, for exposures occurring at lower and varying concentrations over periods of several weeks to perhaps several years, it is not known whether*

military personnel, while also in the presence of other combustion gases, may experience similar additive effects. No relevant chronic or low-level exposure studies were found in the literature.

The recommendations of the United States Army are that (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008):

- The toxic effects of CO and HCN are probably additive, and, therefore, the effects from combined exposures to these chemicals should be assessed as a mixture and not individually.
- Until further findings suggest otherwise, the use of the HQ approach proposed by the Army is reasonable in establishing exposure limits for personnel simultaneously exposed to CO and HCN.
- The use of the CFK equation [Coburn, Forster, and Kane equation] for the prediction of COHb levels related to air concentrations of CO is appropriate (The United States Army uses the Coburn, Forster, and Kane (CFK) equation to estimate the percentage of COHb in the blood of military personnel in armored vehicles based on measurements of CO in the air inside of vehicles; however, it was recommended in 2008 that the Army utilize real-time instantaneous and running average gas monitoring (multiagent monitoring) equipment rather than total reliance on the CFK equation.).
- The CFK equation has not been adequately evaluated in environments with dynamically changing air concentrations, such as in a weaponized armored vehicle.
- The use of an air concentration for HCN in the HQ equation, as opposed to a blood concentration, is reasonable.

The United States Army has also identified a number of important data gaps regarding CO exposures within the modern battlefield/military contexts (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008):

further neurologic studies on sensory and motor performance at lower concentrations of HCN and CO because most studies on the combined toxicity of CO and HCN have been carried out at high concentrations and have focused on lethality and/or incapacitation; this makes it difficult to use those data to extrapolate to low levels of exposures and to assess more-subtle effects of interest to the Army. Finally, the committee recommended that the Army consider concurrent exposures to other chemicals (for example, other combustion products and diesel exhaust), which may have additional effects on the armored-vehicle crew.

24.17.3 Acute toxicity

Importantly, seemingly acute CO poisoning can result from both sudden exposure to high levels of the gas or the cumulative formation of COHb following more prolonged, slower, and more insidious exposures. Clinical signs associated with the acute CO poisoning toxidrome range from nociceptive effects (headache, weakness, upset stomach, vomiting, chest pain, and confusion) to dizziness to death. CO poisoning symptoms have been described as being "flu-like" and the clinical signs in some people can mimic those of alcohol intoxication, that is, some individuals can appear and behave like they are drunk due to ethanol consumption.

Symptoms of CO poisoning begin at approximately 20% COHb and death usually ensues between 50% and 80% COHb (Ryter and Otterbein, 2004). The classical relationship between blood COHb and toxic signs and symptoms is presented in Table 24.5. More detailed studies have also been performed on crew members of the MIR space station (National Research Council, 2008) (Fig. 24.3). The correlation between blood COHb level and clinical manifestations can be unreliable in many patients because of premeasurement delay, initiation of oxygen therapy before blood sampling, and/or concomitant cyanide or other types of poisoning.

Of critical note is that most modern pulse oximetry devices will not detect CO poisoning and are likely to

TABLE 24.5 Relationship of carboxyhemoglobin (COHb) to toxicity in humans following exposure to carbon monoxide.

COHb %	Signs and symptoms
<10	No effects in healthy individuals[a]
10–20	Mild headache, exertional dyspnea, cutaneous vasodilation
20–30	Throbbing headaches, nausea
30–40	Severe headaches, dizziness, visual disturbance, fatigue
40–50	Tachypnea, tachycardia, collapse, syncope
50–60	Coma, convulsions, Cheyne–Stokes respiration
60–70	Cardiorespiratory depression, possible death
>70	Respiratory failure and death

[a]*Symptoms may appear in subjects with cardiovascular disease. Concentration of COHb depends upon the duration of exposure to any concentration of CO in the air and is therefore not included in the table. Exposure to approximately 200 ppm for 2 h results in 10% COHb; ~12,000 ppm CO would produce lethal concentrations of COHb within two or three breaths.*

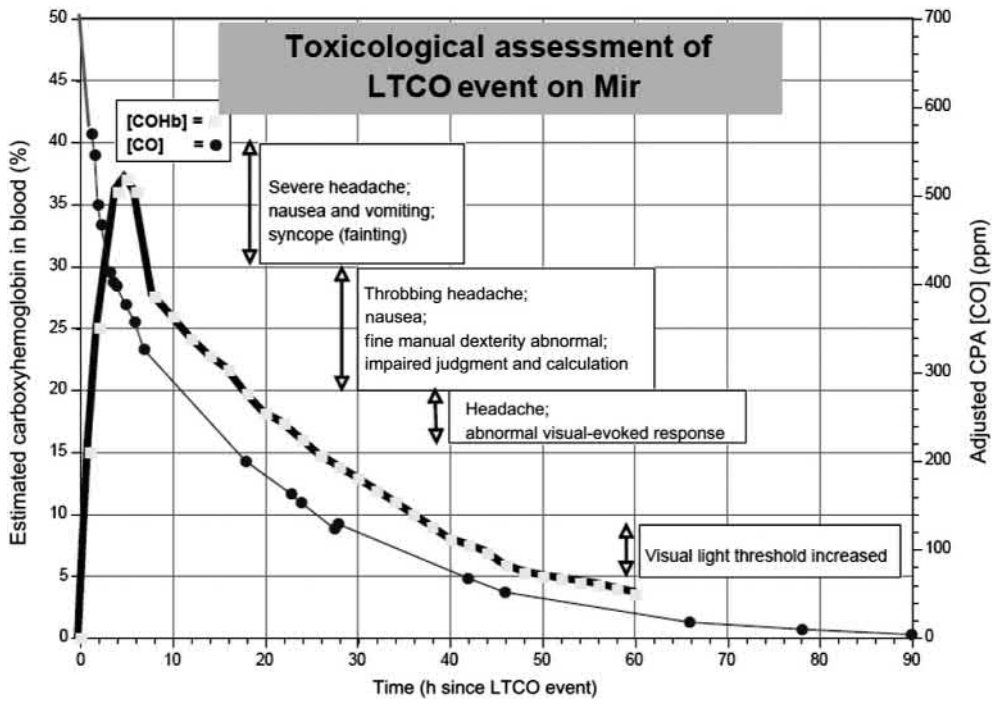

FIGURE 24.3 CO and COHb concentrations and toxic health effects observed on a space station (National Research Council, 2008).

give spurious indications of the level of peripheral blood oxygen saturation. When CO poisoning is suspected, a pulse CO-oximeter or breath CO monitoring should be used. Breath CO monitoring is advantageous in that there is a strong correlation between COHb levels and breath CO concentration.

24.17.4 Delayed (interval) manifestations of acute toxicity

Delayed neurological and neuropsychiatric effects in survivors of serious acute CO toxicity are extremely common and occur within 2−40 days post-exposure at a rate of between 2% and 40% of patients (Mannaioni et al., 2006; Raub and Benignus, 2002; Yoshii et al., 1998; Okeda et al., 1981; Hsiao et al., 2004; Parkinson et al., 2002). Early (acute phase) and late occurring (subacute phase) brain injuries have been noted. In many cases the observed injuries resemble those associated with ischemic-hypoxic central nervous system injuries due to other causes. The most common injuries are symmetrical bilateral basal ganglia abnormalities with the early-phase injuries most commonly being found in the basal ganglia and corpus callosum, and often more clearly, in bilateral globus pallidus. Later occurring injuries tend to be symmetrical deep brain leukoencephalopathies and diffuse inflammations in semicentrum ovale or periventricular brain regions. The neuroclinical manifestations of CO poisoning include memory loss, movement disorders/chorea, Parkinsonian-like syndromes, communication disturbances, depressed mood, dementia, and psychosis.

Visual disturbances after recovery from a period of CO-induced unconsciousness have also been reported (Kelly and Sophocleus, 1978). Moderate to severe CO poisoning can shorten lifespan because of myocardial injury (Henry et al., 2006).

Concurrent CO plus noise exposure has been found to cause hearing loss in humans (Sato, 1966; Morris, 1969; Goto et al., 1972; Makishima et al., 1977) and in animal models (Douglas et al., 1912; Fechter et al., 1988; Liu and Fechter, 1995).

24.18 Typical anatomic pathology findings

The classical gross anatomic pathology findings are cherry red liver mortis with bilateral hemorrhages and/or necrosis in the globus pallidus if the person has survived long enough. Important differential diagnoses for cherry red lividity include thallium poisoning, cyanide poisoning, and hypothermia. The cherry red discoloration is often relatively long-lived compared with cases of cyanide poisoning due to the somewhat slower postmortem reversion of COHb to hemoglobin compared with the postmortem deoxygenation of oxyhemoglobin. The possible presence of cherry red lividity is best evaluated against a white background.

Important microscopic findings, apart from general indications of tissue hypoxia, include foci of ischemic or hemorrhagic necrosis in globus pallidus and perivascular foci of demyelination in deep white matter with sparing of arcuate fibers. At the histopathology level important differentials include methanol poisoning manifesting as necrosis of the putamen and other causes of hypoxia.

24.19 Treatment of carbon monoxide overdose

24.19.1 Oxygen

The fundamental basis of using supplemental oxygen at normal or hyperbaric pressures has been described in detail above and was first elaborated by Haldane (Haldane, 1895, 1922; Haldane and Priestley, 1935). Supplemental oxygen remains the mainstay of treatment of CO poisoning. Hyperbaric oxygen is generally indicated in cases displaying loss of consciousness, ischemic cardiac changes, neurological deficits, significant metabolic acidosis, and COHb greater than 25%. However hyperbaric oxygen therapy remains somewhat controversial due to limited practical evidence of efficacy despite its seemingly valid theoretical justification (likely due to other detrimental modes of action apart from simple tissue hypoxia).

24.19.2 Targeted temperature management

Targeted mild therapeutically induced hypothermia has been used for the treatment of post-cardiac arrest or hypoxic–ischemic brain injury. The experimental underpinnings of the technique are based on the hypothermic inhibition of neurotransmitter (glutamate, dopamine, catecholamines) production, release and reuptake and thus the possible reduction of excitotoxicity and free radical generation (Arrich et al., 2016). Limited retrospective case series data ($n = 227$; Oh et al., 2016) regarding the use of the induced hypothermia in combination with hypobaric oxygen for treatment of patients with severe acute CO poisoning (defined as: history of CO exposure and any of Glasgow coma scale score <9 after acute CO exposure, blood COHb level $>25\%$, and S100B protein level $>0.165\ \mu g/L$) appears to show some beneficial effects. However, a control group was not included in the study.

24.19.3 Sympatholytics and sedation

The theoretical underpinnings for the use of sympatholytics (dexmedetomidine, remifentanil) and sedatives lie in the catecholamine crisis theory of CO poisoning-delayed neurological injury (Oh and Choi, 2015). The objective of sedation and/or sympatholytics is to suppress CNS sympathetic neurological activity. This approach can, at least in theory, be used concurrently with therapeutic hypothermia. There is currently little practical evidence of therapeutic benefit outside of these theoretical underpinnings.

24.19.4 Allopurinol and *N*-acetylcysteine

The basis for this approach is the evidence that some of the toxic effects of CO poisoning are produced by release of reactive oxygen metabolites and xanthine oxidase plays a major role in these processes (Kekec et al., 2010; Sokal and Kralkowska, 1985; Thom, 1992; Zhang and Piantadosi, 1992). Xanthine oxidase is a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase, which is converted to oxidase under ischemic conditions utilizing molecular oxygen in place of NAD and, in the process, generates superoxide radicals and hydrogen peroxide. Allopurinol is a xanthine oxidase inhibitor and *N*-acetylcysteine is a sulfhydryl donor. Limited evidenced of efficacy of these agents against neuronal injury caused by CO has been documented (Kekec et al., 2010; Howard et al., 1987; Thom, 1992). However, substantive clinical trial evidence of efficacy is lacking.

24.19.5 Insulin

Hyperglycemia, which follows stroke or myocardial infarction, worsens the resulting neurological insult. Acute CO poisoning is also characterized by hyperglycemia, which has been found to worsen brain dysfunction in rats (Penny, 1990). Similar observations have also been made in CO-poisoned patients (Pulsinelli et al., 1980). Moreover, neurological complications of CO poisoning in diabetic patients seem worse than in healthy individuals. There is evidence that insulin treatment is effective in reducing neuronal damage caused by stroke or cardiac arrest, and limited evidence of efficacy is available for the treatment of CO poisoning in humans (White and Penny, 1994). Again, substantive evidence of benefit in clinical cases is lacking.

24.20 Acceptable exposure levels within the military context

The United States Army has established a maximum acceptable blood COHb limit of 5% for aviation crews and 10% for all other army personnel (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008). The Army also uses the current US American Conference of Governmental Industrial Hygienists (ACGIH) threshold limit value (TLV) ceiling of 4.7 parts per million (ppm) for inhalable cyanide exposure. Because of the at least

additive effects of CO and cyanide the Army has developed a hazard quotient approach for short-term (less than 30 min) exposures (Committee on Combined Exposures to Hydrogen Cyanide and Carbon Monoxide in Army Operations, 2008):

$$\text{Hazard quotient} = \frac{\text{COHb\%}}{10\%} + \frac{15 - \text{minaverage HCN(ppm)}}{4.7\text{ppm}}$$

Hazard quotient of ≥ 1.0 indicated unacceptable over exposure.

The United States Navy has adopted an approach of assuming that the total irritancy effects of ammonia, chlorine, hydrogen chloride, hydrogen sulfide, nitrogen dioxide, and sulfur dioxide mixtures are additive but not synergistic and uses a cumulative exposure index approach in setting submarine escape action levels (SEALs) (National Research Council, 2002). However, the Navy has called for further research regarding the combined effects of gases that produce various forms of hypoxia, that is, the interactions between hydrogen cyanide, CO, and hydrogen sulfide (National Research Council, 2002). Combined exposures may also occur with acrolein, formaldehyde, hydrazine, methanol, monoethanolamine, and oxygen (National Research Council, 2007). The United States Navy Health Research Center's Toxicology Detachment has proposed two exposure levels, called submarine escape action level (SEAL) 1 and SEAL 2, for CO of 125 and 150 ppm, respectively (National Research Council, 2002, 2007). Importantly SEAL 1 is defined as the maximum concentration of a gas in a disabled submarine below which healthy submariners can be exposed for up to 10 days without experiencing irreversible health effects. The value is based upon a blood COHb level of less than or equal to 15%. SEAL 2 is defined as the maximum concentration of a gas in a disabled submarine below which healthy submariners can be exposed for up to 24 h without experiencing irreversible health effects. Exposures at SEAL 1 and SEAL 2 might produce moderate, reversible health effects but they will not impair the functions of the respiratory system and central nervous system to the extent of impairing the ability of crew members in a disabled submarine to escape or be rescued or perform specific tasks, such as shutting off a valve and using a fire extinguisher. The United States Navy has also set emergency exposure guideline levels (EEGLs) of 180 ppm for 1 h and 45 ppm for 24 h of exposure (National Research Council, 2007). Additionally, a continuous exposure guideline level (CEGL) of 9 ppm has been set for a 90-day exposure duration (National Research Council, 2007).

The United States National Aeronautics and Space Administration has established spacecraft maximum allowable concentrations (SMACs) for CO based upon studies of the performance of crew members of the MIR space station (National Research Council, 2008):

- 1-h SMAC = 425 ppm based on a ≤15% blood COHb level;
- 24-h SMAC = 100 ppm based on a ≤15% blood COHb level;
- 7-day SMAC = 55 ppm based on a ≤15% blood COHb level;
- 30- and 180-day SMAC = 15 ppm based on a ≤2% blood COHb level.

24.21 Defensive measures

Given that inhalation of CO is the major route of exposure of concern under virtually all military circumstances, defensive measures against exposure are potentially relatively simple. Appropriate nonair-supplied respirators and appropriate filters and/or gas scrubbing systems are cheap and readily available. Such systems provide adequate short-term protection from exposures provided that the associated atmosphere is not oxygen-depleted. Air-supplied systems will always be required in oxygen-deprived circumstances, such as those that might be encountered with compartment fires and in low-lying bunker systems, etc. The main limitations of all of these defensive systems are well known and have been recognized since the time of World War I and include: (1) the additional encumbrance of ground troops which limits their mobility and capacity to maneuver at speed; and (2) these defensive systems have a limited period of effective operation, that is, if the affected troops can be contained in such a manner that their gas protection systems become exhausted they then become especially vulnerable to further gas attack.

24.22 Concluding remarks and future directions

Being an inevitable consequence of the combustion of organic materials, battlefield CO exposures, whether deliberately applied or incidental, are likely an ancient phenomenon of warfare dating back to the very beginnings of recorded human history and beyond. Given that combustion is an integral part of the operation of modern military vehicles and weapons systems, incidental CO exposure will continue to be part of the battlefield experience for the foreseeable future. Given the simplicity, ease, and low cost of its production and other factors, the deliberate use of CO as a weapon in particular military circumstances will likely continue. While there is now a very substantial medical and toxicological understanding of the consequences of simple CO exposures there remain

important gaps in the understanding of the implications and role of CO in the effects of complex gas mixtures that are likely to occur in association with modern battlefields and modern weapons systems.

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Chapter 25

Acute cyanide toxicity and its treatment: the body is dead and may be red but does not stay red for long

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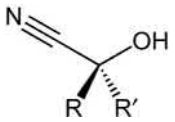
25.1 Introduction: basic terminology and a brief and tragic history of the use and misuse of cyanide

This chapter attempts to provide a basic introduction to the military and paramilitary use of acute cyanide toxicity as a weapon technology. The chapter does not attempt to cover other types of cyanide poisoning phenomena in depth nor does it discuss the various chronic toxicological syndromes associated with cyanide exposure.

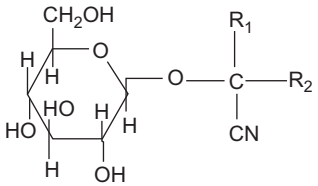
The chemical compounds referred to as cyanides all contain the $-C{\equiv}N$ cyano functional group consisting of a carbon atom triple-bonded to a nitrogen atom. In their inorganic forms cyanides typically exist in the forms of the CN^- anion in solution or its solid salts (commonly sodium cyanide or potassium cyanide). Acidification of cyanide salts results in the formation of the highly volatile liquid hydrocyanic acid (synonyms: hydrogen cyanide, prussic acid), which has the chemical formula HCN and the military designation "AC." Cyanogen chloride ($N{\equiv}C-Cl$) has the military designation "CK."

In military terms, cyanides were historically called "blood agents" (now redundant) since at their time of introduction onto the battlefields of World War I the other members of the suite of available chemical weapons acted at sites of first contact (i.e., the respiratory tract, mucous membranes, eyes, and skin), whereas cyanide produced "systemic effects" that required distribution to the tissues "by blood." In more modern toxicological parlance cyanides are histotoxic hypoxia agents since they inhibit the ability of cells to use oxygen from the bloodstream despite initially normal delivery of oxygen to the cells and tissues via the bloodstream.

Organic compounds that contain an $R-C{\equiv}N$ functional group are typically referred to as nitriles or contain the prefix "cyano." If there are multiple $R-C{\equiv}N$ functional groups in an organic compound it is typically referred to as a cyanocarbon. Cyanohydrins are a type of naturally occurring nitrile that can release hydrogen cyanide. The cyanohydrin functional group consists of a cyano and a hydroxyl group is attached to the same carbon molecule, that is:



Many plants, including some species that are important human and animal food sources, produce cyanogenic glycosides in response to adverse growth conditions and/or for the purposes of chemical defense. These phytotoxins consist of a sugar molecule bonded to a cyanohydrin via its hydroxy functional group (i.e., via a glycosidic bond); for example:



Due to hydrolysis, hydrogen cyanide is released from these molecules following crushing of the plant (e.g., during mastication), digestion, or during food processing.

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The first reported isolation of hydrocyanic acid from Prussian blue [ferric hexacyanoferrate (II)] was by the Swedish chemist C.W. Scheele in 1782, and he was also reported to be its first victim when, in 1786, he accidentally broke a vial of the chemical in the laboratory and died from cyanide vapor poisoning (Ballantyne and Marrs, 1987). However, cyanide and its derivatives have been used as political and military tools since at least the days of ancient Rome (Sykes et al., 1981). Famously, the Roman emperor Nero used cherry laurel water, containing cyanide derived from cyanogenic glycosides during the distillation process, as a political assassination tool. Napoleon III proposed the lacing of soldiers' bayonets with cyanides during the 1870–71 Franco-Prussian War.

During World War I the French army used, largely unsuccessfully, about 4000 metric tons of cyanide in various types of weaponry. The lack of military effectiveness of these weapons has been attributed to several factors: (1) the small size of the munitions could not deliver sufficient amounts of cyanide to cause widescale biological effects under battlefield conditions; (2) hydrogen cyanide's high volatility meant that the agent would quickly evaporate and disperse rather than being persistent on the battlefield; (3) as a killing or maiming agent it had few militarily useful effects below the lethal concentration × time exposure level (i.e., its military useful effects were judged to be essentially "all or nothing"); and (4) German soldiers were adequately equipped to protect themselves from HCN exposure. Mixing HCN with other agents ("Vincennite" consisting of 50% HCN, 30% arsenic trichloride, 15% stannic chloride, and 5% chloroform) did not improve the military effectiveness of cyanide under World War I battlefield conditions.

In 1916, the French experimented with cyanogen chloride. Cyanogen chloride was considered to have the more desirable military properties of being heavier and less volatile than HCN. The effect of cyanogen chloride was similar to HCN. An additional perceived advantage of cyanogen chloride over HCN was that it caused marked lacrimation, rhinorrhea, and increased bronchial secretions in a manner similar to phosgene. Cyanogen bromide (C≡N − Br) was also briefly used in World War I.

The Soviet Union considered the use of cyanide-based weapons possibly as early as the 1920s. Grigory Mairanovsky, the head of the People's Commissariat for Internal Affairs (NKVD) Laboratory 1 from 1938 to 1946 is alleged to have conducted human prisoner experiments on cyanide. By the 1960s declassified US National Intelligence Estimates indicated that the Soviet military had the capacity to produce and maintain militarily effective concentrations of hydrogen cyanide for 10–15 min over a militarily useful area (National Intelligence Estimate Number, 1969).

The story of the development of Zyklon B in pre-Nazi Germany, and its subsequent deliberate, politically motivated misuse by the Nazi government, is of particular note in the history of pesticide toxicology; especially since these agents were developed with the best of intentions at a time of significant need (Robinson, 1971; Baskin and La Cleur, 1998). During World Wars I and II and the interwar period, one of the most feared epidemic public health diseases in the preantibiotic era was typhus, which is caused by *Rickettsia prowazekii*. Typhus is spread by the human body louse *Pediculus humanus*. Another militarily important disease of World War I that was spread by the human body louse is trench fever caused by *Bartonella quintana*. Yet another important, potentially high mortality disease in the preantibiotic era that is spread by the human body louse is relapsing fever caused by *Borrelia recurrentis*. The human body louse, and the diseases transmitted by it, were ubiquitous under the battlefield conditions of World War I; and then subsequently amongst the crowded, impoverished populations of Europe (particularly in Germany) during the interwar period. Thus the control of the human body louse became a major public health and military priority during World War I, the interwar period, and World War II.

During World War I, the interwar period, and World War II fumigation using volatile hydrogen cyanide had become recognized as one of the most effective and reliable ways to control human body louse-borne diseases (and other important food pests) in both military and civilian circumstances. However, the shelf-life of purified liquid hydrogen cyanide is quite short and mishandling of the agent was extremely dangerous. Accordingly, better solutions were actively sought.

During the 1920s scientists working at the Fritz Haber Institute in Germany determined that cyanide could be more safely handled if it was adsorbed into inert, porous materials. Its shelf-life could also be improved if it was coformulated with a chemical stabilizer. These discoveries, the desire to control potentially dangerous louse-borne epidemic diseases, and the desire to reduce pest-associated food loss during a time of widespread relative food insecurity, led to the German production of the now infamous Zyklon B pesticide family (Zyklon in German translates to "cyclone" in English; the letter B stood for *Blausäure*—prussic acid—and was used to distinguish the product from the Zyklon A pesticide product range which used methyl cyanoformate as the active ingredient).

Zyklon B formulations consisted of hydrogen cyanide (about 40% by mass) adsorbed into one of several different absorbents (calcium sulfate or diatomaceous earth were common; use of cyanide absorbents was a legal requirement in Germany at the time), one of several

different cautionary eye irritants (the inclusion of which, notably, was not a legal requirement in Germany at the time), and one of several different stabilizers. Cautionary eye irritants were used since it was well understood by this time that a portion of the population were functionally anosmic to the classic bitter almond smell of cyanide vapor. In a further attempt to ensure safe use, 200 g of the Zyklon B formulation was packed in metal cans that could only be opened with a special tool (Fig. 25.1). Based on the number of different patents for the Zyklon B formulation family (held by Deutsche Gesellschaft für Schädlingsbekämpfung mbH aka Degesch; whose first director, somewhat ironically given that several members of his family were subsequently eliminated using Zyklon B during the holocaust, was Fritz Haber) there was no chemically simple approach to the need for chemical stabilization.

For its time, Zyklon B fumigation proved highly effective and beneficial in reducing human body louse-borne disease, for controlling various vertebrate and invertebrate pests, and for minimizing pest-associated food loss. In many cases (including at Holocaust sites) legitimate Degesch-designed tunnel/chamber delousing facilities were used. The correct civilian use of Zyklon B in the manner that was originally intended was reputedly, for the era, relatively safe and likely saved many human lives.

FIGURE 25.1 Canisters of Zyklon B found in the Dachau concentration camp. *United States Holocaust Memorial Museum; Provenance William and Dorothy McLaughlin. Source Record ID: Collections: 2005.442.*

By 1936 Degesch, was part owned by Degussa AG (which, ironically, also produced the plasticizer and anti-graffiti coating used in the 2005 Berlin Holocaust Memorial), and IG Farben. These companies produced the Zyklon B without eye irritant (perfectly legal in Germany at the time) that was subsequently sold to the German army and the Schutzstaffel via the companies of Tesch & Stabenow GmbH (Testa) and Heerdt-Linger (Heli).

Dissatisfied with the use of carbon monoxide and shooting as methods of quick and efficient systematic mass murder, Rudolf Hoess, the Schutzstaffel commandant of the Auschwitz extermination camp from 1940 to 1943, and his deputy Karl Fritzsch began experimenting with the use of Zyklon B on humans. At the time Zyklon B fumigation was legitimately being used within the German prisoner of war and forced labor camps for human body louse and other pest control. Based on Hoess's experiments that involved the gassing of 600 Soviet prisoners of war and 250 sick prisoners judged to be incapable of forced labor, Zyklon B was judged to be the quickest and most efficient mass murder method available. The pesticide formulation, which had been developed with the best of intentions, subsequently became the chosen method of mass murder at Auschwitz (and across the other Nazi killing centers and concentration camps), and was eventually adopted across the entire Nazi "final solution" operations.

Following his capture, Rudolf Hoess claimed that he introduced Zyklon B because he considered it to be more humane than killing by shooting, brutalization, and starvation. In his memoirs he claimed Zyklon B gassings were reassuring to him because his victims supposedly suffered less than if they had been shot. However, subsequent historical investigations demonstrated that the use of Zyklon B was more based on practical expedience rather than any pretensions of "humanitarianism." It is of note that some Zyklon B formulations produced a blue stain on areas of contact. These stains remain visible on the walls of the gas chambers in the Nazi killing centers (Fig. 25.2).

Japanese military forces also actively used chemical weapons during World War II. It has been claimed that cyanide-based munitions were used in some of the approximately 2000 chemical attacks instigated by Japanese military forces in China during the 1931–45 period (Yan 2014). The degree of military effectiveness of these weapons is unknown. The Japanese army chiefs of staff also allegedly ordered the killing of over 400 prisoners and staff using food laced with potassium cyanide as part of a cover up of activities by the Pingfan BC/Unit 731 warfare facility in China during August 1945 (Harris, 2002).

FIGURE 25.2 Interior view of a gas chamber at the Majdanek concentration/extermination camp in Poland (post-liberation) showing blue staining on the walls due to the use of Zyklon B. *Panstwowe Muzeum na Majdanku. Copyright: Public Domain.*

The use of cyanide-based weapons may have reoccurred during the 1980–88 Iran-Iraq War. One of the perhaps better known incidents occurred during the Iranian offensive against the Kurdish city of Halabja. While a mixed chemical weapon attack involving mustard gas, a nerve agent (possibly tabun or sarin or VX or a combination of these agents) and cyanide was reported at the time, it has subsequently been claimed that most of the fatalities were caused by cyanogen chloride (Baskin and Rockwood, 2002; Segal, 1986; USDIA, 1988). Iranian physicians have claimed that some of the victims of the attack were successfully treated using inhalation of amyl nitrite and injection of thiosulfate; however, definitive diagnoses of acute cyanide poisoning were never established. Subsequent investigators have noted that people affected by the attack appeared to have been killed by surprise while going about their normal daily activities and have noted that the predominant skin discoloration seen amongst the dead was blue (cyanosis) and not red (however, while considered "classical," red skin discoloration is not always, in practice, a regularly observed clinical sign of acute cyanide poisoning). On this basis there have been alternative claims that most of the deaths during the attack were due to a nerve agent (likely tabun) and that hydrogen cyanide poisonings were secondary to the hydrolytic or thermal decomposition of this agent. Other reputed cyanide-based chemical weapon attacks during this conflict include those on the Iran-Iraq border towns of Sardasht, Marivan, Sarpol-e Zahab, Gilan-e-gharb, and Oshnavieh.

Cyanide sources have also been used as part of religion-associated chemical terrorism and/or mass murder-suicide. The archetypical example of cyanide use with religion-associated mass murder-suicide were the events at the Peoples Temple Agricultural Project ("Jonestown") in Guyana in 1978. Doctors associated with the sect reputedly tested the efficacy of cyanide as a killing agent on pigs based on their claim that the metabolism of this species closely resembles that of humans.

Another example of religious/cult-associated attacks that may have utilized cyanide is the Aum Shinrikyo attacks in Tokyo in 1995 (Sidell, 1996). Cyanide gas precursor compounds were found in several subway restrooms following these attacks. Allegedly, cyanide was also added to the explosives used in the first attack on the World Trade Center in New York (Brennan et al., 1999). Al-Qaeda and other organizations with claimed affiliation with the Osama bin Laden terrorist franchise also allegedly experimented with the use of hydrogen cyanide (Pita, 2015).

The use of cyanides as a tool of political and military assassination is also well known. Famously, cyanide-laced food and drink failed to kill Grigori Rasputin during an assassination attempt in 1916. In 1954, the Soviet KGB agent Nikolai Khoklov was ordered to assassinate the Russian anti-communist exile Georgiy Okolovic in Frankfurt, Germany, using an electrically operated, suppressed cyanide gun disguised as a cigarette packet. Khoklov did not go through with the assassination, subsequently defected to the United States, and provided details of the plot during a broadcasted interview. The murders of two Ukrainians, Rebet (1957) and Bandera (1959), were committed by a Soviet agent in Munich using a gas pistol containing cyanide (Anders, 1963).

The American Mafia hitman and serial killer Richard Kuklinski reputedly favored the use of cyanide by injection, in food, as an inhaled aerosol spray, or by skin contact as an assassination tool. In his view it killed quickly, was not messy, could in some cases be mistaken for an acute cardiopulmonary death and, at that time, was reputedly hard to detect during routine toxicology testing.

The toxicology of acute cyanide poisoning and its treatment have been extensively discussed by many authors (Ballantyne and Marrs, 1987; Baskin et al., 1997; Borowitz et al., 1992; Gonzales and Sabatini, 1989; Marrs et al., 1996; Salkowski and Penney, 1994; Vennesland et al., 1981; Way, 1984). However, the key modes of action of acute cyanide poisoning have only been fully elucidated relatively recently (Borowitz et al., 2001; Gunasekar et al., 1996; Sun et al., 1997).

25.2 Sources of exposure

The use of cyanide for "purely" military purposes has been, overall, relatively limited and fortunately largely ineffective for the reasons described above. However, as a tool of assassination and mass killing, it has proven to be highly effective. Most human acute poisoning cases

involve civilians and most nonhomicidal cases are due to fires or industrial accidents. Human–cyanide interactions remain very common because of its widespread industrial use. Worldwide industrial consumption of cyanide is estimated to be 1.5 million tons per year, with occupational exposure accounting for many cases of acute cyanide poisoning (Cummings, 2004; Logue et al., 2010; Coentrão and Moura, 2011). Acute cyanide poisoning may occur from a broad range of exposure sources (summarized in Table 25.1).

25.3 Toxic levels of cyanide

It is not easy to determine the lethal dose of cyanide in humans. Haber's law is a reasonable predictor of mortality associated with acute cyanide poisoning, that is, the dosimetry follows the concentration multiplied by time function. The maximum permissible concentration for HCN in humans is 11 mg/m^3 (Ballantyne, 1974). Taken orally, the fatal dose of HCN to adults is estimated to be 50–100 mg; for KCN it is estimated to be 150–250 mg (DuBois and Geiling, 1959). On a mass per unit body weight basis the fatal dose of HCN is estimated to be in the 0.7–3.5 mg/kg range (Hallstrom and Moller, 1945). However, victims ingesting as much as 3.0 g of KCN have been saved with immediate treatment (Van Heijst et al., 1987). Table 25.2 shows that inhalation of HCN at a concentration of 300 mg/m^3 (approximately 270 ppm) will be immediately fatal, whereas at 20–40 mg/m^3 mild symptoms will appear after several hours of exposure (Rumack and Newball, 1983). Victims with a blood cyanide level of 2.5–3.0 μg/mL frequently succumb to respiratory cessation within 20–30 min of exposure but some may survive up to 3 h (Ballantyne, 1974; Van Heijst et al., 1987). Oral ingestion of cyanide may also produce rapid onset of symptoms, typically because the doses encountered far exceed the minimal lethal dose. Studies of experimental animals have shown that absorption of cyanide decreases with alkaline stomach pH due to the relatively high pKa of HCN. However, based on simple Hendersen–Hasselbalch calculations a quite high gastric pH would be required to significantly reduce absorption.

TABLE 25.1 Various possible sources of cyanide poisoning.

Fire smoke	Smoke generated after combustion of silk, polyurethanes, polyacrylonitriles, nylon, melamine resins, plastics, etc., in accidents including industrial, residential, car, aircraft, ship fires
Industrial exposure	Plastics production, dyeing, printing and photography, fumigation of pesticides/rodenticides, synthetic rubber production, fertilizer production, metal polish, tanning in leather industry, electroplating, metallurgy, paper and textile manufacture
Drugs	Sodium nitroprusside, laetrile, succinonitrile
Dietary	Cassava, lima beans, linseed, bamboo sprout, macadamia nuts, hydrangea, Rosaceae family (plum, peach, pear, apple, bitter almond, cherry), *Sorghum* species (Johnson grass, sorghum, Sudan grass, arrow grass), *Linum* species (flax, yellow pine flax)
Others	Cigarette smoking, phencyclidine synthesis, ingestion of nail polish remover, suicide, homicide, terrorist attack, chemical warfare, capital punishment

TABLE 25.2 Toxicity of hydrogen cyanide by inhalation.

Concentration		Effects
mg/m^3	ppm	
20–40	18–36	Slight symptoms after several hours
50–60	45–54	Endurable for 20–60 min without effect
120–150	110–135	Very dangerous (fatal) after 30–60 min
150	135	Lethal after 30 min
200	181	Lethal after 10 min
300	270	Immediately lethal

Normally most cyanide is absorbed within 2−3 h of ingestion (Ryan and Viccellio, 1998). However, substances such as nitrile compounds and cyanogenic glycosides like amygdalin from plants require enough time for conversion to cyanide before they can produce symptoms of toxicity (Ryan and Viccellio, 1998).

Laetrile (often called by its misnomer "vitamin B17") is a claimed complementary/alternative "pseudo-medicine" for cancer treatment. It is synthesized from the cyanogenic glycoside amygdalin, which is most commonly encountered in the seeds of stone fruits from *Prunus* sp. One gram of laetrile contains the equivalent of 60 mg of cyanide, and each laetrile tablet may contain up to 100 mg of laetrile. A 12- to 18-tablet laetrile overdose is sufficient to produce severe metabolic acidosis and convulsions in humans (Ellenhorn et al., 1997).

The lethal toxicity of HCN and its alkali salts by different routes for different species of animals and sexes has been discussed elsewhere (Ballantyne and Lindstrom, 1984; Ballantyne and Marrs, 1987). A study performed using rabbits revealed the following order of decreasing toxicity of KCN administered by different routes: intravenous > intramuscular > intraperitoneal > oral > instillation into conjunctival sac > percutaneous (Ballantyne and Lindstrom, 1984). The comparative oral LD_{50} values of KCN for different species of animals are given in Table 25.3. Rabbits are generally regarded as being more susceptible than rats and mice.

25.4 Detection and estimation of cyanide

Determination of cyanide or its metabolites in biological fluids is necessary for forensic, clinical, military, research, and veterinary purposes. The choice of analytical methods depends on a variety of factors including sensitivity, specificity, rapidity, convenience, facilities, and expertise. The selection of biological sample, time of sampling, time to analyze, storage conditions, and interfering substances are other factors that influence the choice of analytical methods (Troup et al., 1987; Jackson et al., 2014). There are several convenient and sensitive methods for measuring cyanide in biological fluids, but many of them have limitations. Some of these methods are summarized here.

TABLE 25.3 Acute lethal toxicity of KCN by oral route for different species of animals.

Species	Sex	LD_{50} (mg/kg)
Mouse	Male	8.50
	Male	12.5
Rat	Male	10.0
	Female	7.49
	Female	14.1
Rabbit	Female	5.82

One of the most common procedures includes diffusion and trapping of cyanide in the alkaline media before colorimetric analysis in pyridine−pyrazolone mixture (Epstein, 1947). This method was subsequently modified for microdiffusion analysis of cyanide (Feldstein and Klendshoj, 1954). This procedure is widely used during the treatment of cyanide intoxication and thiosulfate is known to interfere in the colorimetric estimation, which was subsequently resolved (Morgan et al., 1979). A rapid (approximately 20 min), specific, and sensitive spectrophotometric method for whole blood cyanide assay has also been developed (LaForge et al., 1994). All these procedures are based on the König reaction, which starts with the production of cyanogen chloride. A spectrofluorometric determination of cyanide and thiocyanate based on a modified König reaction in a flow-injection system was also reported with detection limits of 30 nM for both anions (Tanaka et al., 1992). Spectrophotofluorometry is also a convenient, sensitive method provided that previous microdiffusion has been performed to isolate and concentrate the cyanide. The fluorometric methods using pyridoxal are more sensitive and require fewer and more stable reagents than the colorimetric method (Takanashi and Tamura, 1970). However, sodium thiosulfate is known to interfere with the chemical conversion of the fluorophore and it is possible to circumvent the interference by using acetate buffer (pH 5.2) as the acidifying agent. Another fluorescent method with an advantage over pyridoxal uses para-benzoquinone, and this method is not known to have any extraneous interference (Guilbault, 1976).

The potentiometric determination of cyanide using ion-selective electrodes has become yet another very popular technique because it is a convenient, rapid, and sensitive method of analysis (Frant et al., 1972). Microdiffusion of biological samples containing cyanide is recommended before potentiometric determination. The use of a cyanide ion-selective electrode in combination with the Conway microdiffusion method for the measurement of cyanide concentrations in human red blood cells (RBCs) and plasma was reported with remarkable recovery of cyanide (Yagi et al., 1990). Ion chromatographic determination of sulfide and cyanide in real matrices using pulsed amperometric detection on a silver electrode was reported by Giuriati et al. (2004). Ion chromatography with pulsed amperometric detection has also been used to determine cyanide in urine and saliva samples (Jaszczak et al., 2017).

The measurement of HCN directly by gas chromatography has also been reported, but this method lacks sensitivity with most detectors (Valentour et al., 1974). Gas chromatographic techniques are not widely used for measuring cyanide because other methods are more convenient. A simple and sensitive method was devised for determining cyanide and its major metabolite, thiocyanate, in blood using an extractive alkylating agent (pentafluorobenzyl bromide). The detection limits of cyanide and thiocyanate were 0.01 and 0.003 μmol/mL, respectively (Kage et al., 1996). Rapid quantitation of cyanide in whole blood by automated headspace gas chromatography was performed on clinical samples from fire victims. This method could detect a wide concentration of blood cyanide (30−6000 μg/L) in approximately 17 min (Calafat and Stanfill, 2002).

A direct and sensitive isotope dilution−mass spectrometry determination of blood cyanide by headspace gas chromatography was developed with a detection limit of 0.3 μmol/L (Dumas et al., 2005). This method was also compared with other techniques in a round-robin exercise. Cyanide can also be measured by indirect atomic absorption spectrometry where a metal−cyanide complex is formed and is then extracted in organic solvent to determine the metal content (Manahan and Kunkel, 1973). An original high-performance liquid chromatographic−mass spectrometric (HPLC-MS) procedure was developed for the determination of cyanide in whole blood. The limits of detection and quantitation were 5 and 15 ng/mL, respectively. Also, several other methods including HPLC, using post-column derivatization with *o*-phthalaldehyde (Sumiyoshi et al., 1995), capillary electrophoresis with fluorescence detection, polyphenol oxidase/clay biosensors, capillary electrophoresis microchip, ICT-based probes (Badugu et al., 2005), and microchemiluminiscence have been reported for different environmental or biological samples. However, their utility to detect cyanide in blood samples is yet to be ascertained. A technique to detect cyanide currently utilized by water treatment facilities was used to rapidly detect concentrations of cyanide in the clinically important range. The CYANTESMO test strips accurately and rapidly detect cyanide of more than 1 μg/mL. A paper test for cyanide (CYANTOSNO) in whole blood is now commercially available in the United States (Ellenhorn et al., 1997).

25.5 Toxicokinetics of cyanide

25.5.1 Absorption

Oral absorption of cyanide is rapid, and toxic effects can occur within minutes. When salts of cyanide are ingested hydrochloric acid in the stomach causes the release of HCN. HCN is a weak acid with a p*K*a of 9.2 and is thus mostly in its more lipophilic, readily absorbable, unionized form within the acid environment of the stomach. HCN is also generally rapidly absorbed at other sites of exposure (respiratory tract, eyes) (Ballantyne, 1974; Borowitz et al., 2001; Ellenhorn et al., 1997; Ryan and Viccellio, 1998).

In rabbits, cyanide introduced into the conjunctival sacs was quickly absorbed in significant quantities to produce systemic toxicity (Ballantyne, 1983). Predictably, dermal absorption is somewhat slower. However, it is notable that at the typical skin pH range of 4−5.5, HCN's pKa implies that it is mostly in its more easily absorbed, relatively lipophilic, nonionized state. The large skin surface area also likely facilitates absorption. Nitriles are more readily absorbed through the skin, but the onset of toxicity is delayed due to the time required for them to be metabolized and cyanide released.

25.5.2 Distribution

After absorption, distribution is rapid (Sylvester et al., 1983) with an apparent volume of distribution of about 40% of total body weight. This implies distribution mostly to the extracellular fluid volume and a lack of apparent tissue sequestration. Consistent with this are the findings that after oral poisoning a significant amount of cyanide was traced in the brain, blood, kidney, stomach wall, liver, and urine (Ansell and Lewis, 1970). Cyanide is rapidly transported in the body by blood and approximately 60% is bound to plasma proteins with a RBC: plasma ratio of 100:1 (Ellenhorn et al., 1997; Ryan and Viccellio, 1998). Following acute exposure the plasma elimination half-life of cyanide is estimated to be 14.1 min (Egekeze and Oehme, 1979).

25.5.3 Elimination

Cyanide is rapidly detoxified in mammals. To determine the effect of species on cyanide metabolism, toxicokinetics of cyanide was studied in rats, pigs, and goats after oral dosing of KCN (Sousa et al., 2003). The study showed that metabolism of cyanide and its main metabolite, thiocyanate, is species-linked, with goats being the most sensitive to the toxic effects of cyanide. The detoxification rates following intravenous dosing of humans, dogs, guinea pigs, and rabbits are 0.017, 0.020, 0.04, and 0.008 mg/kg body weight per minute, respectively (Hinwich and Saunders, 1948; NIOSH, 1975). The major pathway of cyanide detoxification (approximately 80%) is through enzymatic transulfuration to thiocyanate (SCN^-), which is subsequently excreted in urine (Lang, 1933). This reaction is catalyzed by rhodanese (thiosulfate cyanide sulfur transferase; EC.2.8.1.1). Rhodanese uses a precursor like thiosulfate as a source of sulfane sulfur

(divalent ionized sulfur bound to another sulfur atom). The endogenous supply of this substance is very limited. Thus detoxification of large doses of cyanide depends on an exogenous supply of thiosulfate (Westley et al., 1983). However, cyanide detoxification by this pathway is often debated because rhodanese is located principally in the mitochondria and penetration of the cell wall and mitochondrial membrane by thiosulfates is very slow (Bhat et al., 1983). It is presumed that the sulfane sulfur binds first to the serum albumin to yield a sulfane sulfur albumin complex that eventually reacts with cyanide to form thiocyanate (Westley et al., 1983; Way, 1984). In normal metabolism of cyanide, the serum albumin−sulfane complex may be the primary detoxification mechanism (Sylvester et al., 1983).

Unlike many other chemical warfare agents, cyanide is present in blood, urine, and expired breath (Lundquist et al., 1988). Cyanide and its metabolites are eliminated from the body by several mechanisms. After 3 h, approximately 90% of injected cyanide has been shown to be eliminated in the dog model (Sylvester et al., 1983). A small amount of administered cyanide is excreted in the urine and via the lungs after being incorporated into cyanocobalamin (vitamin B_{12}), oxidated to formate and carbon dioxide, and incorporated with cystine (Ballantyne and Marrs, 1987). Other minor pathways of detoxification include enzymes like mercaptopyruvate sulfur transferase, thiosulfate reductase, and cystathionase γ-lyase or disulfide cystine, and 2-iminothiazolidine-4-carboxylic acid (2-ICA) or its tautomer, 2-aminothiazolidine-4-carboxylic acid (2-ACA) (Baskin et al., 2004; Wood and Cooley, 1956). Cyanide reacts with cystine to produce β-thiocyanoalanine, which spontaneously undergoes ring closure to form 2-ICA and 2-ACA, depending on the pH in the cells (Borowitz et al., 2001).

25.6 Mechanism of action

The toxic effect of cyanide is attributed predominantly to the production of cellular anoxia after inhibition of the metal-containing enzymes. The critical interaction appears to be the inhibition of the terminal respiratory chain enzyme, cytochrome oxidase a_3 (containing iron), within the mitochondria. The enzyme is essential for the production of adenosine triphosphate (ATP). As a result, aerobic oxidative metabolism and phosphorylation are impaired, leading to cellular hypoxia. The pyruvate that is produced can no longer be used and is now reduced to lactate. The shunt from aerobic to anaerobic metabolism leads to profound lactic acidosis (Solomonson et al., 1981). Cyanide toxicity may not be ascribed solely to a single biochemical lesion but rather to a complex phenomenon. Cyanide reacts with several metalloenzymes, carbonyl groups of different enzymes, coenzymes, and substrates, resulting in

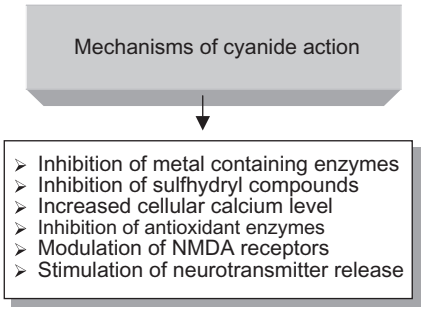

FIGURE 25.3 Mechanisms for the toxic manifestations of cyanide exposure.

inhibition of normal activity. Cyanide also interacts with sulfhydryl compounds like cystine, mercaptopyruvate, reduced glutathione, and oxidized glutathione to form different complexes (Borowitz et al., 1992; Way, 1984). Cyanide also strongly interacts with iron in protein molecules, inhibiting enzymes including carbonic anhydrase and succinic dehydrogenase (Ballantyne and Marrs, 1987). Formation of cyanhemoglobin by interaction of cyanide with ferric iron abolishes the ability of hemoglobin to carry oxygen (Way, 1984). The classical mechanism of cyanide toxicity is summarized in Fig. 25.3.

Cyanide is regarded as a selective neurotoxin and its toxicity has frequently been associated with elevated levels of cellular calcium (Johnson et al., 1986), inhibition of antioxidant defense enzymes in the brain (Ardelt et al., 1989), and generation of reactive oxygen species (ROS), leading to lipid peroxidation (Ardelt et al., 1994; Kanthasamy et al., 1997). Whether or not these effects are fully "de novo" effects of cyanide or are, at least in part, downstream secondary effects due to the failure of cellular energy generation in a high energy demand tissue is not fully elucidated.

Cyanide increases intracellular calcium through activation of voltage-sensitive calcium channels (Johnson et al., 1987), direct redox modulation and "excitotoxin-like" enhancement of *N*-methyl-d-aspartate (NMDA) receptor function (Patel et al., 1992; Sun et al., 1997), and mobilization of intracellular calcium stores (Yang et al., 1997). It was proposed that elevated cytosolic calcium activates proteases, which in turn convert xanthine dehydrogenase to xanthine oxidase. In the presence of oxygen, xanthine oxidase can catalyze the formation of superoxide radicals that initiate lipid peroxidation (Fig. 25.4) (Cheung et al., 1985). Overall, it appears that oxidative stress plays a crucial role in cyanide-induced neurotoxicity (Ardelt et al., 1989; Kanthasamy et al., 1994).

Modulation of NMDA receptor has been widely implicated in cyanide-induced neurotoxicity. Nitric oxide and ROS generation after NMDA receptor activation was found to mediate cyanide-induced neurotoxicity (Gunasekar et al., 1996). Several other studies also

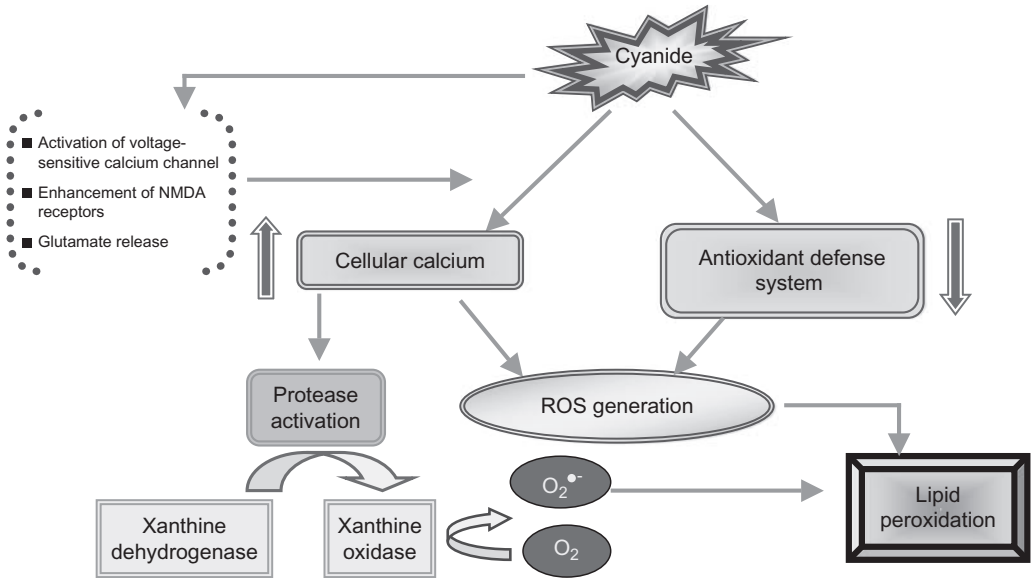

FIGURE 25.4 Pathway of cyanide-induced oxidative stress. O_2, Oxygen; $O_2^{\bullet-}$, superoxide anion; *ROS*, reactive oxygen species.

showed activation of NMDA receptors during cyanide toxicity (Patel et al., 1992; Sun et al., 1997). Cyanide can also stimulate release of glutamate from intracellular stores, resulting in elevation of cytosolic Ca^{2+} through NMDA receptor activation. There is also evidence that cyanide may interact directly with the NMDA receptor to enhance NMDA receptor-mediated Ca^{2+} influx (Patel et al, 1994; Sun et al., 1995). It was further shown that cyanide selectively interacted with NMDA subunits, possibly by formation of thiocyanate adduct with a cysteine residue located in the NR1 receptor subtype (Arden et al., 1998).

Cyanide is also known to interact with cystine to produce 2-ICA and 2-ACA, and the former is responsible for memory loss, convulsions, and loss of consciousness (Bitner et al., 1991). Cyanide can also produce dopaminergic toxicity characterized by loss of dopaminergic neurons in the basal ganglia that is accompanied by impaired motor function (Kanthasamy et al., 1994). It has been suggested that the convulsive effects of cyanide are due to changes in the levels of dopamine (Cassel, 1995). Cyanide is also known to stimulate neurotransmitter release in both the central and peripheral nervous systems (Kanthasamy et al., 1991). Modulation of protein kinase C, calmodulin, and nitric oxide (NO)-dependent cyclic guanosine monophosphate-dependent enzymes and ATP depletion may also contribute to the induction of convulsions. Other biochemical processes that may mediate or at least influence cyanide toxicity include severe lactic acidosis, mitochondrial ADP ribosylation, and hyperammonemia (Borowitz et al., 1992).

Another important aspect of acute cyanide toxicity is its cardiotoxic manifestations (Baskin, 1991; Borowitz et al., 1992). The acute, pro-arrhythmogenic cardiac effects of cyanide likely explain way its effects can, in some cases, mimic an acute "heart attack-like" or an acute cardiopulmonary failure-like clinical presentation, that is, sudden collapse with cardiopulmonary failure and a few premonitory clinical signs. Apart from redox effects with mechanisms similar to those described above perhaps one of the key effects of acute cyanide poisoning on the heart is disruption of calcium homeostasis and excitation–contraction coupling in cardiac excitable cells (Cheung et al., 1985). Its effects on the physiological functions of calcium in these cells are complex and include multiple different excitation–contraction abnormalities, depolarization of resting membrane potential, reduced action potential amplitude, prolonged action potential duration, depolarization of mitochondrial membrane potential, and suppression of depolarization-activated K^+ currents.

25.7 Diagnosis and clinical features of cyanide poisoning

The signs and symptoms of acute cyanide poisoning are often nonspecific and vary in both time and intensity depending on the scale of exposure (Ballantyne, 1974, 1987; Ellenhorn et al., 1997; Hall et al., 1987; Nicholson and Gupta, 2012; Way, 1984). The signs and symptoms of acute cyanide poisoning reflect cellular hypoxia and can be fairly nonspecific. The onset of the toxidrome depends on the dose, route, and duration of exposure. In general, clinical signs and symptoms correlate with the whole blood cyanide level (Table 25.4). However, the

TABLE 25.4 Whole blood cyanide levels and clinical signs and symptoms.

Whole blood cyanide		Signs and symptoms
μg/mL	μmol/L	
0–0.5	8–20	No symptoms
0.5–1.0	20–38	Tachycardia, flushing, headache, hyperpnea, dizziness
1.0–2.5	48–95	CNS depression including giddiness, tachypnea, nausea, vomiting feeling, suffocation, confusion
2.5–3.0	95–114	Respiratory depression, convulsion, coma, cyanosis, apnea, circulatory collapse, fixed dilated pupils
≥3.0	114	Death

overall severity of poisoning does not correlate particularly well with serum cyanide concentration.

Early clinical manifestations due to effects on the CNS include anxiety, headache, giddiness, dizziness, confusion, and mydriasis. Cardiovascular effects can be somewhat variable but all derive from cyanide-induced hypoxia and depression of the cardiovascular system. Bright (cherry red) retinal veins due to elevated venous PO_2 have been suggested as a possibly clinical useful early clinical observation. Other early cardiovascular effects may include palpitations, diaphoresis, dizziness, flushing, tachycardia, and hyperventilation. Initially, increased cardiac output and blood pressure may occur due to catecholamine release. However, eventually vasodilatation, hypotension, reduced cardiac ionotropic effects, and shunting of blood to the brain and heart will ensue. At an overall level cyanide exposure triggers sinoatrial node depression, negative cardiac ionotropic effects, and a pro-arrythmogenic state. This leads to unstable hemodynamic states, atrioventricular blocks, ventricular arrhythmias, bradycardia, heart block, cardiac arrest, and death.

Later clinical manifestations of acute cyanide poisoning also include loss of consciousness, seizures, paralysis, coma, and hypoventilation/apnea. Classically, skin and mucous membrane color has been described as being "cherry red" due to the presence of increased venous PO_2 despite the presence of tissue hypoxia. However this classical description is not reliable since skin and mucous membrane color can appear normal to somewhat ashen. Perhaps the most critical feature is a *lack of cyanosis or disproportionately mild cyanosis for a given level of cardiovascular/respiratory failure* due to high venous PO_2.

Based on a review of 21 cases of acute cyanide poisoning associated with suicidal ideation the three leading manifestations of the toxidrome were sudden loss of consciousness (about 71% percent of cases), severe metabolic acidosis (about 66% of cases), acute cardiopulmonary failure (about 43% of cases), and anoxic encephalopathy (about 29% of cases; Yen et al., 1995). In this case series, diabetes insipidus-like syndromes were considered to be an ominous clinical development in patients with cyanide-associated encephalopathy.

Laboratory tests suggestive of cyanide intoxication include arterial blood gases (metabolic acidosis with normal PO_2), serum electrolytes (elevated anion gap), central venous percent O_2 saturation (elevated), calculated arterial percent O_2 saturation (normal), and measured arterial percent O_2 saturation (decreased). Serum lactate levels greater than 8 mmol/L are associated with acute cyanide poisoning.

Quantitative determination of cyanide in whole blood, urine, gastric contents and tissues, and plasma thiocyanate levels are also important (Ballantyne, 1983). Serum concentrations of cyanide greater than 0.5 mg/L are typically associated with acute cyanide poisoning. Unfortunately, these tests take several hours and the results may not be available to the clinician during the acute phase of poison management. The Prussian blue reaction can be used as a quick bedside test that can qualitatively detect cyanide in gastric aspirate; however it is prone to false-positive results (Hall et al., 1987; Lee-Jones et al., 1970).

There are no reliable pathognomonic anatomic pathology lesions that can be used to definitively diagnose acute cyanide toxicity. If present, cherry red skin/mucous membrane discoloration tends to fade relatively quickly after death (unlike with carbon monoxide poisoning where the discoloration is more persistent due to the presence of carboxyhemoglobin), especially if venous blood is exposed to normal air. This is because the potentially high venous PO_2 levels typically dissipate fairly quickly after death.

Animal experiments indicate that anatomic pathology lesions are often in the central nervous system (CNS), particularly within white matter (Ballantyne, 1974). However, these findings are usually a general manifestation of anoxic encephalopathy and are not necessarily pathognomonic of acute cyanide poisoning.

25.8 Treatment of cyanide poisoning

The onset of toxicity after cyanide poisoning is very fast. The prognosis of the victim depends on termination of further exposure, supportive care, and institution of immediate and aggressive specific treatment. Early diagnosis and clinical information would enhance the chances of recovery. Critically, first responders and treatment teams are potentially at risk of cyanide poisoning when attempting to recover and treat patients. Degassing of cyanide vapors from poisoned patients as well as environmental sources are important forms of exposure under such circumstances. The use of appropriate personal protective equipment is strongly recommended.

The first principle of therapy is termination of further exposure, which can be facilitated by the following: (1) remove the victim from the contaminated atmosphere; (2) apply a protective mask to the patient as soon as possible to prevent further inhalation; (3) remove any liquid on skin or clothing as soon as possible; and (4) remove all contaminated clothing, rinse skin with soap and copious amounts of water or water alone if there is liquid on the skin (Baskin et al., 1997). Despite poor binding to activated charcoal, animal data suggest that a single oral dose of the material at a rate of 1 g/kg body weight with a maximal dose of 50 g may decrease mortality. Single dose activated charcoal treatment is unlikely to be of much benefit if dosing is delayed for 2−3 h.

25.8.1 Antidotal therapy

Antidotal therapy should be commenced as soon as possible. Hydroxocobalamin is the antidote of choice because it is regarded as being effective and does not further compromise cellular hypoxia and acidosis. The standard dose is 5 grams given intravenously (i.v. over 15 min; be aware that this antidote turns urine dark red: this is not due to myoglobinuria).

Various cyanide antidotes have been proposed over a period of many years (Table 25.5). Cyanide antidotes have been classified into three main groups based on their mechanism of action: methemoglobin inducers, sulfur donors, and cobalt compounds. The definitive treatment of cyanide poisoning differs in various countries because of different medical practices and guidelines. The safety and efficacy of all the antidotes are still being debated. However, it is important to note that hydroxycobalamin has now largely replaced the older antidotal protocols.

25.8.2 Methemoglobin inducers

The basic aim of rapid detoxification of cyanide is prevention or reversal of inhibition of cytochrome oxidase by cyanide. This is usually facilitated by providing a large pool of ferric iron in the form of methemoglobin to complex cyanide. Cyanide preferentially competes with the

TABLE 25.5 Supportive therapy and specific antidotal therapy for acute cyanide poisoning.

	Agents	Drug category and action
Supportive therapy	100% oxygen or hyperbaric oxygen	Oxygen: Potentiates the efficacy of nitrite−thiosulfate therapy
	Sodium bicarbonate	Alkalizing agent: Corrects lactic acidosis
	Diazepam	Anticonvulsant: Depresses CNS activities
	Epinephrine	Sympathomimetic: Improves coronary and cerebral blood flow, corrects anaphylactoid reactions
Specific antidotal therapy	Amyl nitrite	Methemoglobin inducers: Converts hemoglobin to methemoglobin, which binds with cyanide to form cyanmethemoglobin. In the presence of sodium thiosulfate cyanide is excreted as thiocyanate
	Sodium nitrite	
	4-Dimethylaminophenol	
	Sodium thiosulfate	Sulfur donors: Facilitates enzymatic conversion of cyanide to thiocyanate
	Dicobalt edentate	Cobalt compounds: Forms stable metal complexes with cyanide
	Hydroxocobalamin	

Fe^{3+} of methemoglobin as compared with that of cytochrome oxidase and eventually binds with the former to form cyanmethemoglobin. Thereby, the activity of inhibited cytochrome oxidase is restored (Baskin et al., 1992). The various methemoglobin inducers used as cyanide antidotes are discussed below.

25.8.3 Amyl nitrite

Although inhalation of amyl nitrite as a first aid measure for cyanide poisoning has been known for many years, its efficacy as a methemoglobin inducer is often disputed because of its inability to generate methemoglobin more than 6% (Jandorf and Bodansky, 1946). Approximately 15% of methemoglobin is required to challenge one LD_{50} of cyanide (Van Heijst et al., 1987). The protective effect of amyl nitrite is attributed to its vasodilatory effect, which can reverse the early cyanide-induced vasoconstriction (Van Heijst et al., 1990). Artificial ventilation with amyl nitrite broken into Ambu bags has been reported as a life-saving therapy in cyanide-poisoned dogs before induction of significant levels of methemoglobinemia (Vick and Froehlich, 1985).

25.8.4 Sodium nitrite

Sodium nitrite is the most prevalent drug for cyanide poisoning. It takes approximately 12 min to generate approximately 40% of methemoglobin after intravenous administration of the recommended dose (Van Heijst et al., 1987). Despite this delay in inducing a significant level of methemoglobinemia, reasonable protection offered by sodium nitrite can be attributed to its vasodilatory effects (Van Heijst et al., 1990). A major drawback with sodium nitrite is that it causes serious cardiovascular embarrassment, particularly in children (Berlin, 1970). Because methemoglobinemia impairs oxygen transport, sodium nitrite cannot be recommended for fire victims when concomitant exposure of HCN and carbon monoxide usually occurs. Because carbon monoxide also reduces the oxygen-carrying capacity of blood, administration of sodium nitrite would further aggravate the underlying hypoxic condition. Sodium nitrite is also contraindicated for individuals with glucose-6-phosphate dehydrogenase-deficient red cells because of the possibility of serious hemolytic reactions (Way, 1984). Excessive levels of methemoglobin are known to be reverted by intravenous administration of 30 mL of 1% methylene blue solution (Van Heijst et al., 1987). In the United States, the Lilly Cyanide Antidote Kit (manufactured by Eli Lilly and Company, Indianapolis, IN) includes 10 mL ampoules of 3% sodium nitrite solution and 50 mL of 25% sodium thiosulfate solution. The kit also contains amyl nitrite encased in glass "pearls," which are meant to be broken so the drug could be inhaled. In India, a similar kit is manufactured by Troikaa Pharmaceuticals Ltd (Thiol).

25.8.5 4-Dimethylaminophenol

The relatively slow rate of methemoglobin formation by sodium nitrite prompted the development of rapid methemoglobin formers like aminophenols. 4-Dimethylaminophenol (DMAP) is the treatment of choice for cyanide poisoning in Germany. Administered intravenously, a dose of 3.25 mg/kg DMAP was reported to produce methemoglobin levels of 30% within 10 min and 15% methemoglobinemia was attained within 1 min without any immediate effect on the cardiovascular system (Kiese and Weger, 1969). However, there are differences in individual susceptibility to DMAP that may result in undesirable levels of methemoglobin even after normal therapeutic doses (Van Dijk et al., 1987). Intramuscular injection of DMAP results in local abscess and fever. Its clinical utility remains limited because of its other toxicological implications like nephrotoxicity.

25.8.6 Sulfur donors

After the initial therapy of methemoglobin inducers, cyanide has to be converted to thiocyanate, which is eliminated in urine. This enzymatic detoxification of cyanide is facilitated by a sulfur donor like sodium thiosulfate. The mechanism of this reaction was discussed previously under the elimination of cyanide. High tissue oxygen markedly potentiates the effects of this reaction. In cases in which methemoglobin formation is not desirable, sodium thiosulfate together with oxygen alone is sufficient. The utility of thiosulfate alone is limited because of its short biological half-life and its small volume of distribution (Sylvester et al., 1983). Also, thiosulfate is contraindicated in patients with renal insufficiency because the thiocyanate formed may cause toxicity (Van Heijst et al., 1990).

25.8.7 Cobalt compounds

The cobalt ion, which forms a stable metal complex with cyanide, is an effective therapeutic agent against cyanide poisoning (Evans, 1964; Hillman et al., 1974; Linnell et al., 1987). Various cobalt-containing compounds known to antagonize cyanide poisoning are discussed below.

25.8.8 Dicobalt edetate (Kelocyanor)

Dicobalt edetate chelates cyanide as cobalticyanide. This drug is known to antagonize cyanide more quickly than the nitrites, but its clear superiority has not been established. Intravenous administration of 300 mg of dicobalt edetate in glucose solution is the current treatment of choice in France and the United Kingdom. Serious side effects like vomiting, urticaria, anaphylactoid shock, hypotension, and ventricular arrythmias have been reported in patients receiving Kelocyanor (Van Heijst et al., 1990).

25.8.9 Hydroxocobalamin (Cyanokit)

With the exchange of the hydroxy group of hydroxocobalamin (vitamin B_{12a}) for cyanide, nontoxic cyanocobalamin (vitamin B_{12}) is formed (Hall and Rumack, 1987). An injectable solution of hydroxocobalamin (5 g in water) has been used in France and Germany. In France, a 4 g hydroxocobalamin solution in 80 mL of sodium thiosulfate has also been used (Van Heijst et al., 1990). EMD Pharmaceutical Company has produced a lyophilized packaging of 2.5 g of hydroxocobalamin that can be readily reconstituted in a 100-mL sodium chloride solution. This product is pending FDA approval. It has been in use in Europe since 1996 as the Cyanokit. Hydroxocobalamin is also used in other countries, including Sweden, Denmark, Spain, Japan, and Hong Kong. Sodium thiosulfate or hydroxocobalamin has also been recommended for empiric treatment of cyanide poisoning (Hall et al., 2007). There are several disadvantages in the clinical use of this drug. It has a relatively short half-life because it decomposes in light, and the dose required to counter cyanide poisoning is quite large. Also, recorded side effects include anaphylactoid reactions and acne (Van Heijst et al., 1990). However, in a case study, clinical laboratory data did not show any evidence of toxicity (Borron et al., 2007).

25.8.10 Supportive therapy

The details of supportive therapy are presented in Table 25.5. The single most important form of supportive treatment is 100% oxygen. This includes mechanical airway support, artificial ventilation with 100% oxygen. If amyl nitrite is being used as an antidotal treatment it can be included in the breaking circuit (e.g., an Ambu bag containing the contents of two 0.6-mL ampoules of amyl nitrite) in association with cardiac monitoring (Van Heijst et al., 1987). Anecdotal evidence suggests that hyperbaric oxygen augments the protective efficacy of nitrite–thiosulfate therapy (Goodhart, 1994).

Lactic acidosis resulting from anaerobic metabolism and convulsions should be treated with intravenous administration of sodium bicarbonate and diazepam, respectively (Baskin et al., 1997; Van Heijst et al., 1990).

25.9 Concluding remarks and future directions

Currently, cyanide is considered not to be an important chemical weapon agent. It has been used, with moderate success, as an assassination agent. However, the threat of its possible use in local terrorism cannot be overlooked. Cyanide toxicity is mainly ascribed to its ability to inhibit cytochrome oxidase, an end-chain enzyme of cellular respiration. Numerous new mechanisms of action of cyanide unfold the complex toxic phenomena occurring at cellular and molecular levels. Many of these mechanisms have been the target for pharmacological interventions. Hydroxocobalamin has become the cyanide antidote of choice in recent times.

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Chapter 26

Methyl isocyanate: the Bhopal gas

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26.1 Introduction

Methyl isocyanate (MIC), the smallest, most reactive, and most toxic member of the isocyanate family, was generally unheard of until December 2−3, 1984, when nearly 40 metric tons of this deadly chemical leaked out of the Union Carbide India Limited (UCIL) pesticide plant at Bhopal within a period of 45−60 min. Bhopal became a "city of death," in the words of *India Today* (December 30, 1984). The journal *Nature* (Opinion, 1984) wrote: "... the anguish vividly carried round the world by the television cameras seems not to have matured into the anger, even hysteria, there would have been had the accident occurred on the edge of a European city—or in Connecticut [the site of Union Carbide's US headquarters]."

Prior to the Bhopal disaster, there had been only one scientific report on MIC toxicity (Kimmerle and Eben, 1964); this led *Lancet* (Editorial, 1984) to comment: "In a year's time we will have learnt a lot more about MIC—at an appalling price." The Bhopal disaster evoked immense interest amongst journalists, scientists, the corporate world, lawyers, social activists, and the Indian government (Chemical & Engineering News, 1985; Varma and Saxena, 1986). This chapter describes the physicochemical characteristics of MIC and its toxicity, and how the Bhopal disaster took place and how such disasters can be prevented in developing countries like India, which lacks rigorous safety protocols for hazardous chemicals.

The population of Bhopal in 1984 was 800,000. The Union Carbide plant was within 1 kilometer of the Bhopal railway station and not far from the city's two large hospitals; densely populated slums lay across the road just a few hundred meters from the factory. The toxicity of a chemical, barely tested in animal models, was now suddenly being observed as it acted on 200,000 people, as well as on animals and plants living in Bhopal. There is no precise information on how many people died in the immediate aftermath of the disaster; usually a figure of 2500 is quoted, but our estimate based on projecting deaths in a sample survey (Varma, 1987) is that about 8000 people died. Most of the deaths happened between 24 and 48 h after the discharge of MIC, which would not have been the case if the culprit had actually been hydrogen cyanide (HCN), as was thought initially (HCN acts within minutes). A British medical student on her elective at a Bhopal hospital wrote: "The dead and dying arrived by the truckload, others came by rickshaw or were carried by relatives. For some, the effort of the journey itself proved too much, and they died soon after arrival" (Sutcliffe, 1985).

Soon after the Bhopal accident, the government of India promised to conduct a comprehensive study on the acute and long-term effects of exposure to MIC. The Indian Council of Medical Research (ICMR) released approximately $2 million for research; a cohort of 80,021 gas-exposed people and 15,931 controls were registered, but only 16,860 exposed subjects and 5741 controls could be contacted in 2010 (Sharma, 2013). Researchers were not recruited to study all aspects of toxicity. No definite criteria were set to make a quantitative assessment of the exposure to MIC; the best estimate has remained the distance from the Union Carbide factory (Dhara et al., 2002). A number of laboratories in the United States and United Kingdom initiated research on MIC, but their interest faded quickly.

26.2 The making of a disaster

For days after the disaster, no one was sure how the accident had happened. A detailed account of how MIC escaped from Tank E-610 of UCIL was ultimately provided by Stewart Diamond in *The New York Times* on January 28, and this report also was corroborated by others (Varadarajan et al., 1985; Varma and Saxena, 1986). Although it is highly unlikely that an accident

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would occur exactly in the way it happened in Bhopal, the accidental escape of hazardous chemicals is not uncommon. The US Environmental Protection Agency (EPA) had recorded 28 instances of minor leaks of MIC from the Union Carbide plant in Institute, West Virginia, between 1980 and 1984, and a leak of aldicarb oxime from the same plant on August 11, 1985, sent 200 people to hospitals.

According to Diamond's article, water entered the pipes on the floor of the factory during routine cleaning, which took place without placing safety slips at the joints. It would seem that the floor of a chemical factory was cleaned with no more precautions than are taken when cleaning the platform of an Indian railway station. The water reached Tank E-610. The exothermic reaction between the water and MIC increased the temperature of the tank, converting liquid MIC into gas. The increase in pressure forced open the vent valve, allowing most of the MIC to escape as gas. Various safety measures to neutralize MIC, such as caustic soda scrubbers, were either inadequate or completely nonfunctional. Even if safety measures had been in perfect working order, they were not designed to handle such a large leak. MIC gas, being almost twice as heavy as air, settled on the ground, and affected all living beings in the area. Undoubtedly, the lack of rigorous safety procedures in the maintenance was the cause of the accident.

Fortunately, MIC stored in the other two tanks (E-611 and E-619) was not affected. The Indian Council of Scientific and Industrial Research (CSIR) decided that the safest way to dispose of the remainder of the MIC was to convert it into carbaryl, the pesticide for which the factory had been set up to produce. This exercise was termed "Operation Faith," and it was extensively covered by Indian and international media. Operation Faith ended safely on December 22, 1984, without any further mishap. Nonetheless, the disaster frightened the people of Bhopal so much that despite all assurances by the Indian government, almost half of the population left the town; some never returned to Bhopal.

The Bhopal disaster clearly indicates that the accident would not have occurred if all necessary precautions had been taken in the maintenance and operations of UCIL. Union Carbide had sent a team of American experts in May 1982, and they pointed out many lapses in the operation of the plant. However, the company failed to act on their suggestions. In addition, after making a number of visits to the plant, local journalist Rajkumar Keswani warned of the impending disaster 4 months before the accident.

MIC was used in the United States, Germany, and Japan, but it was stored in Bhopal in much greater quantities than in any of these other places. The question of whether the operation of hazardous industries (like the chemical industry) can be both safe and profitable is debatable. What is not debatable, however, is that safety must remain the top priority. Following a cyclohexane explosion in Flixborough in 1974, an Advisory Committee on Major Hazards was set up in the United Kingdom, and the European Economic Council Directive of 1982 was triggered by the Sevesco accident of 1976 in Italy. However, the Bhopal disaster of 1984, which was far worse than the Flixborough and Sevesco accidents (in fact, it was the worst in history), has not led to additional regulations outlining corporate and state responsibilities, despite the strong case for global monitoring of potentially toxic materials (Sriramachari and Chandra, 1997).

26.3 Chemistry and toxicokinetics of isocyanates

As stated previously, MIC is a member of the isocyanate family of chemicals. The high chemical reactivity of isocyanates is central to their commercial use, but it is also a key element of their toxicity. No clinical use of isocyanates has so far been demonstrated.

26.3.1 Chemistry of isocyanates

Organic isocyanates were first synthesized in 1849. Isocyanates (Table 26.1) are highly reactive heterocumulene

TABLE 26.1 Commonly used isocyanates.

Isocyanates	MW	LC_{50} (ppm)[a]	Ceiling (ppm)[b]
Methyl isocyanate (MIC)	57	5.1	0.02
Hexamethylene diisocyanate (HDI)	168	55.9	0.02
Toluene diisocyanate (TDI)	174	49.0	0.02
Isophorone diisocyanate (IPDI)	222	28.5	0.02
Diphenylmethane diisocyanate (MDI)	250	36.0	0.02
Dicyclohexylmethane diisocyanate (SMDI)	262	–	0.01
1,5 Naphthalene diisocyanate (NDI)	210	–	0.02

[a] *LC_{50} (lethal concentration killing 50% of experimental animals) values are after 4 h exposure.*
[b] *To convert ppm to mg/m^3, divide it by (24.4/MW); see, review by Varma and Saxena (1986).*

chemicals. The general structure of isocyanates is R—N=C=O, which is distinct from that of cyanate (N=C—O—H). The reactivity of organic isocyanates is due to the strain in the cumulative double bonds (—N=C=O) of isocyanates (Varadarajan et al., 1985).

Most of the commercially used isocyanates are diisocyanates, and R is an aromatic ring. MIC is an exception; its structure is $H_3C—N=C=O$. The physicochemical properties of MIC differ from those of other isocyanates (Lowe, 1970). Because of the high chemical reactivity of MIC with alcohols, it serves as an intermediate in the production of carbaryl, a pesticide. Diisocyanates are primarily used for the manufacture of polyurethanes.

26.3.1.1 Synthesis of methyl isocyanate

MIC ($CH_3N=C=O$) can be synthesized using different reactions. The commercial synthesis of MIC by Union Carbide, Bayer, and Dupont is described next.

At Union Carbide Corporation, Bhopal, India:

$$CH_3NH_2(\text{monomethyl amine}) + COCl_2(\text{phosgene}) \rightarrow CH_3N=C=O + 2HCl$$

At Bayer, Germany:

$$CH_3NH\tilde{a} - CO - NHCH_3(\text{dimethyl urea}) + (C_6H_5O)_2CO(\text{diphenyl carbamate}) \rightarrow CH_3N=C=O + \text{by products}$$

At Dupont, the United States:

$$CH_3 - NH - CHO(\text{methyl formamide}) + O_2CH_3N=C=O + H_2O$$

MIC was produced and stored in Bhopal, whereas CO and phosgene, which also are required for the production of carbaryl, were not stored; rather, they were produced and utilized immediately. In the United States and Germany, MIC is produced as needed; it is not stored.

The various steps in the production of carbamate pesticide at Bhopal were as follows (Varadarajan et al., 1985):

1. Petroleum coke (2C) was reacted with oxygen to produce 2CO.
2. CO and chlorine were reacted to produce phosgene ($COCl_2$).
3. Phosgene and methylamine (CH_3NH_2) were reacted to form methylcarbamoyl chloride ($CH_3NHCOCI$) plus HCl.
4. Methylcarbamoyl was then pyrolyzed to yield MIC ($CH_3N=C=O$) and HCl.
5. Finally, MIC was reacted with a slight excess of α-naphthol in the presence of a catalyst in carbon tetrachloride solvent to produce the desired pesticide carbaryl.

In the system used in Bhopal, the stored liquid MIC was transferred through pipes to charged pots weighing 1 metric ton and under 16 psi of pressure of nitrogen. These charged pots were connected to two reactors, where MIC and α-naphthol reacted to produce carbaryl. The charging of α-naphthol was done by dissolving it in carbon tetrachloride at approximately 50°C in the presence of a trimethylamine catalyst. The reaction between MIC and α-naphthol is exothermic. The temperature was maintained at 70°C for efficient production of carbaryl.

$$CH_3N=C=O + a - \text{naphthol} \rightarrow 1 - \text{naphthyl} - N - \text{methyl carbamate (carbaryl)}$$

The alternative method to make carbaryl involves reacting α-naphthol with phosgene to generate α-naphthol chloroformate. Then, α-naphthol chloroformate reacts with methylamine to produce carbaryl.

26.3.1.2 Physicochemical reactions with methyl isocyanate

MIC can interact with a large number of molecules, as well as with itself. Indeed, 21 products were identified (Varadarajan et al., 1985) and almost 10 unidentified chemicals (Sriramachari, 2004) were detected leaking from Tank E-610. An MIC trimer, as well as other metabolites of MIC such as dimethyl isocyanurate and 2,4-dione of MIC, were identified in autopsies of Bhopal victims. Reaction of MIC with water is important because it will occur whenever MIC comes into contact with the body or the environment, as happened in Bhopal. It is important to note that while excess water can neutralize MIC, only small quantities of water are enough to generate heat during the reaction, which leads to the vaporization of MIC, and this is what happened in Bhopal. Some important interactions of MIC are enumerated next.

1. Polymerization (self-addition of many MIC molecules);
2. Trimerization: 3 ($CH_3N=C=O$) → trimethyl isocyanurate;
3. Dimerization is common with aromatic isocyanates, but is rare with aliphatic isocyanates like MIC;
4. Additive reactions with molecules containing reactive hydrogen species, which migrate to the nitrogen of MIC;
5. General reaction of MIC with molecules containing hydroxylic groups:

$$CH_3N=C=O + ROH \rightarrow CH_3NHCOOR \text{ (urethane)}$$

6. Reactions with water:

$$CH_3NCO + H_2O \rightarrow CH_3NH_2(MMA) + CO_2$$

$$2(CH_3NCO) + H_2O \text{ (excess)} \rightarrow CH_3NHCONHCH_3(DMU)_+ CO_2$$

$$3(CH_3NCO) \text{ in excess} + H_2O \rightarrow CH_3NHCON\,(CH_3)\,CONHCH_3(TMB) + CO_2$$

$$4(CH_3NCO) + H_2O \rightarrow DMI + (CH_3)2NH\ (DMA) + CO_2$$

7. Reaction with DMA:

$$CH_3NCO + DMA \rightarrow (CH_3)2N - CO - NH - CH_3(TMU)$$

8. Reaction with alcohols and phenols (used by UCIL to produce carbaryl pesticide):

$$CH_3NCO + \alpha - naphthol \rightarrow$$
$$1 - naphthyl - N - methyl\ carbamate\ (carbaryl)$$

9. Reaction with primary and secondary amines:

$$CH_3NCO + R - NH_2 \rightarrow CH_3 - NH - CO - NH - R$$

10. Reaction with nitrates and nitrites:
    The reaction of MIC with nitrates and nitrites, which are normally present in water, can yield carcinogenic nitrosamines;
11. Decomposition into HCN at temperatures in the range of 427°C–548°C at 55–300 torr (Blake and Ijadi-Maghsoodi, 1982);
12. Reaction with HCN at normal temperatures, leading to the formation of other cyanides (Slotta and Tschesche, 1927).
13. Reactions with body constituents.

In general, interactions between isocyanates and endogenous molecules are reversible (Tse and Pesce, 1978). MIC has been shown to cause a greater interaction with macromolecules than aryl isocyanates (Brown et al., 1987). Reversible conjugation of isocyanates with glutathione (Slatter et al., 1991), which occurs both spontaneously and enzymatically, may have been the mechanism of distributing MIC molecules to different parts of the body and the reason behind its diffuse toxicity profile (Baillie and Slatter, 1991; Pearson et al., 1991). MIC can act as a hapten, which leads to the generation of antibodies in both animals and humans (Karol et al., 1987). MIC can carbamylate macromolecules (Segal et al., 1989). MIC has been shown to be an effective antisickling agent in vitro; it combines with α-amino groups of hemoglobin and thus increases its oxygen-binding affinity (Lee, 1976).

26.3.1.3 Quantification of methyl isocyanate

Measurement of MIC in the workplace requires the collection of samples with special tubes and then reacting it with an amino-based reagent such as 1-(2-methoxyphenyl) piperazine (2MP) or other similar substances (von Zweigbergk et al., 2002). Sampling under controlled experimental conditions can be done using gas-tight syringes. Several techniques have been used for the quantitative analysis of MIC. Methods used by Ferguson et al. (1986) and by ourselves (Varma et al., 1987) used a Perkin-Elmer Model 3920 gas chromatograph equipped with a nitrogen-phosphorus detector. The absolute retention time for MIC under these conditions was approximately 1 min and sensitivity of the method was 0.8 ng MIC.

26.4 Mechanism of death following exposure to methyl isocyanate

The immediate effect of exposure to MIC in Bhopal was lacrimation, choking sensations, and difficulty in breathing, followed in many cases by death. An important element of the fatalities in Bhopal was a lag period of several hours between the exposure to MIC and death (Paintal, 1986; Varma and Saxena, 1986). Most deaths occurred more than 24 h after the exposure. Delayed death was also observed in experimental animals exposed to MIC (Alarie et al., 1987; Bucher et al., 1987; Varma et al., 1988). It would thus appear that the lethal effects of MIC were caused by pulmonary complications. It was also found that a single exposure to MIC can produce long-lasting pulmonary complications (Ferguson and Alarie, 1991; Kamat et al., 1992; ICMR, 2004).

The pattern of death in experimental animals (i.e., rats, mice, and guinea pigs) following exposure to MIC was biphasic and similar to that observed in Bhopal. Even excessive concentrations (3506 ppm for 15 min) of MIC were not lethal to rats in a 10 min period. Exposure to MIC caused a significant decrease in body weight within 24–48 h (Varma et al., 1988), suggesting substantial loss of body fluid. In the only controlled experiments on humans, Kimmerle and Eben (1964) noted that subjects could not tolerate 21 ppm MIC even for a few seconds.

26.5 The cyanide controversy

In the chaos that prevailed in Bhopal following the disaster, a number of journalists, social activists, and even some scientists contended that the culprit was HCN, not MIC. HCN poisoning leads to cherry-red venous blood (which showed up in the autopsies of the victims) because oxygen is not being used by the tissues. Sodium thiosulfate is a known antidote for HCN, and because there were reports that the victims benefited from this type of treatment, that further supported the theory of HCN involvement. Also, MIC can be converted into HCN at high temperatures and pressure (Sriramachari, 2004). However, this was not borne out by the sum total of evidence that emerged. The physicochemical properties and toxicity profiles of MIC and HCN are shown in Table 26.2.

Cyanide is an instant killer at a certain dose level. However, deaths by pulmonary edema after a lag period (Paintal, 1986) and other toxicities observed in Bhopal victims (Varma, 1987) are not known to be caused by

TABLE 26.2 Properties of MIC and HCN.

Property	MIC	HCN
Molecular weight	57	27
Appearance	Liquid	Liquid/gas
Boiling point (°C)	39.1	25.7
Vapor density (air = 1)	1.97	0.95
Threshold limit value (ppm)	0.02	10.0
Lethal level 1 h exposure (ppm)	3.0	100
Concentration immediately fatal (ppm)	Undetermined	>270
Antidote	None	Sodium thiosulfate
Long-term effects	Many	None identified

cyanide. Some cyanide is present in most individuals, and more so if exposed to smoke and fire. Any beneficial effect of sodium thiosulfate could be explained by environmental exposure to cyanide, especially because a majority of the victims were slum dwellers who may have been using fires to keep their homes warm, and many were chronic smokers. Cherry-red blood could have resulted from the formation of carboxyhemoglobin from exposure to atmospheric carbon monoxide.

Under controlled experimental conditions, sodium thiosulfate did not reduce the toxicity of MIC (Alarie et al., 1987; Bucher et al., 1987; Varma et al., 1988). Finally, HCN is a fast killer at concentrations > 100 ppm (Goldstein et al., 1968), and the concentrations of MIC that were achieved in Bhopal would not have killed anyone if the gas had been HCN. Moreover, HCN could not descend to the ground because, unlike MIC, it is lighter than air. Moreover, there is definite evidence of long-term effects in survivors of the Bhopal disaster (Bucher, 1987; Dhara and Dhara, 2002; Dhara et al., 2002; Sriramachari, 2004), whereas a single dose of cyanide is not known to produce such effects (Goldstein et al., 1968). Sodium thiosulfate is well known to be an effective antidote against cyanide poisoning, and it should be administered almost immediately for that purpose, rather than several days or even months later (Chen and Rose, 1956; Goldstein et al., 1968).

26.6 Toxicity of isocyanates

Commonly used isocyanates include toluene diisocyanate (TDI), methylenediphenyl diisocyanate (MDI), naphthalene diisocyanate (NDI), and hexamethylene diisocyanate (HDI). All isocyanates are toxic (Rye, 1973) to varying degrees; TDI seems to be the most toxic.

The National Institute of Occupational Safety and Health in the United States projected as early as 1978 that approximately 50,000−100,000 workers would be exposed to these chemicals within 2 years (NIOSH, 1978). The routine method of exposure of workers to isocyanates is by inhalation, and their toxicity is greater following inhalation than following oral ingestion; isocyanates that produce both pulmonary and sensory irritation are more toxic than those that cause only sensory irritation (Weyel et al., 1982).

26.7 Toxicity of methyl isocyanate

Experimental research on the toxicity of MIC vapor on rats, mice, rabbits, and guinea pigs, as well as on human volunteers, was first reported in 1964 (Kimmerle and Eben, 1964). For the next 20 years, though, no follow-up studies were done. It is to the credit of Kimmerle and Eben (1964) that their stringent observations were confirmed by all the studies that followed the Bhopal disaster of 1984. It is tragic that the toxicity of poisons is tested on humans during wars, more often than not by the most developed countries. It is equally unfortunate that one finds out about human toxicity of chemicals during industrial accidents or as a consequence of environmental neglect. In this sense, Bhopal offered the most expansive opportunity to observe and investigate the toxicity of MIC on such a large scale on humans, as well as livestock and vegetation. Surprisingly, however, most of the obvious questions raised in the aftermath of the Bhopal disaster have not been answered.

Although human toxicity to MIC has only been observed following inhalation, animal experiments reveal that it is also toxic following injection; this contradicts the prediction by the visiting American team soon after the disaster that MIC is so reactive that it would be

destroyed upon contact with the body. Metabolites of MIC are also toxic (Varma and Guest, 1993).

For the sake of simplicity, data on the toxicity of MIC on humans and animals are presented separately in the rest of this chapter. It is worth mentioning, however, that almost all the data derived from animal studies seem to confirm what has been observed in humans in Bhopal.

26.7.1 Toxicity of methyl isocyanate in animal models

26.7.1.1 Mortality

Barely 4 h after the disaster on December 3, 1984, the streets of Bhopal were littered with dead animals—790 buffalo, 18 bullocks, 84 calves, 270 cows, 483 goats, 90 dogs, and 23 horses (Varma and Saxena, 1986). According to autopsy reports, the dead animals showed swollen livers and lymph nodes, bloated digestive tracts, engorged blood vessels, edema, necrosis in lungs with blood clots, and congested hearts and kidneys (Varma and Saxena, 1986). House flies survived, however, but it is not known why.

Kimmerle and Eben (1964) estimated an LC_{50} value of 5 ppm in rats following a 4 h exposure, and 21 ppm following a 2 h exposure. Unlike with cyanide, death followed several hours after MIC exposure and continued for up to 18 days. In later studies, it was found that 10 min exposure to as much as 3506 ppm was not immediately lethal (Dodd et al., 1986), although guinea pigs died during exposure to high concentrations (i.e., greater than 500 ppm). In general, deaths following exposure to MIC occur 1−2 days later, and a second phase of mortality follows after a week or more (Alarie et al., 1987; Bucher et al., 1987; Varma et al., 1988). Guinea pigs are more sensitive to MIC toxicity than rats (Dodd et al., 1986).

26.7.1.2 Pulmonary toxicity

Kimmerle and Eben (1964) reported that MIC caused lacrimation, mucosal irritation, and pulmonary edema in rats, mice, rabbits, and guinea pigs. Other studies also found that MIC causes both sensory and pulmonary irritation; if death did not ensue, the recovery from these pulmonary effects was very slow to occur (Ferguson et al., 1986; Alarie et al., 1987). Exposure to MIC caused concentration-dependent degenerative changes in bronchiolar and alveolar epithelium in rats and guinea pigs, resulting in the plugging of major airways and atelectasis (Nemery et al., 1985; Fowler et al., 1987), increase in lung weight (Bucher et al., 1987; Stevens et al., 1987), pulmonary (Bucher et al., 1987), and olfactory epithelial necrosis, airway obstruction, and compromised cardiopulmonary function (Tepper et al., 1987) in surviving animals.

In a retrospective study of 4782 Bhopal gas victims and 1190 control subjects, De (2012) found a much higher risk of developing obstructive pulmonary complications in younger subjects (age 10−29 years) exposed to MIC than older ones (age 30−60 years).

26.7.1.3 Ocular toxicity

As was the case in humans, lacrimation has also been found to be one of the earliest effects of MIC vapor in experimental animals (Bucher et al., 1987; Varma et al., 1988). However, exposure of rats to 3, 10, or 30 ppm MIC for 2 h (which approximates the situation in Bhopal) was not found to cause any damage to the cornea, although copious lacrimation was observed up to 3 months. Similar findings have been reported in mice (Boorman et al., 1987). On the other hand, exposure of lens explants to MIC in vitro has been shown to cause opacity (Harding and Rixon, 1985). It is very likely that profuse lacrimation acted as a protective mechanism, by chemically inactivating MIC.

26.7.1.4 Reproductive toxicity

Exposure of mice on day 8 of gestation (gestation period 19 days) to 2, 6, 9, and 15 ppm MIC for 3 h or 1−3 ppm for 6 h on days 14−17 of gestation caused concentration-dependent fetal loss and maternal mortality (Varma, 1987; Varma et al., 1987); lengths of different fetal bones were significantly reduced in mice following exposure to 9 and 15 ppm MIC (Varma, 1987). MIC also caused maternal and fetal toxicity in rats; pregnancy loss accompanied a sudden decrease in progesterone, although it could not be determined which of the two events occurred first (Varma et al., 1990). Given the extensive nature of MIC toxicity, it is difficult (if not impossible) to determine if MIC-induced reproductive toxicity is a direct effect on the conceptus or a consequence of general toxicity; however, several observations indicate the possibility of a direct effect.

Radio-labeled MIC rapidly reaches the fetus (Ferguson et al., 1988). Intraperitoneal injection of MIC also caused reproductive toxicity of a similar magnitude as with inhalation (Varma et al., 1990). Moreover, MIC metabolite methylamines also produced reproductive toxicity without other obvious effects on pregnant mice. Of the three amines tested, monomethylamine, dimethylamine, and trimethylamine, the last was most toxic in vivo, as well as in mouse embryos in culture (Varma and Guest, 1993). Interestingly, administration of trimethylamine during mouse pregnancy resulted in stunting of male but not female progeny (Guest and Varma, 1993), similar to the effect reported years later in Bhopal

victims (Ranjan et al., 2003). Another metabolite of MIC, *S*-(*N*-methylcarbamoyl) glutathione (GSH) and MIC metabolite trimethylamine exerted marked toxicity on cultured mouse embryos, as well as yolk sac and limb bud (Guest et al., 1992).

26.7.1.5 Immunotoxicity, genotoxicity, and carcinogenic effects

MIC has been found to generate specific antibodies in guinea pigs following both inhalation and subcutaneous injections (Karol et al., 1987). MIC was found to be genotoxic in rats (Dutta et al., 1988) and caused dose-dependent increases in sister chromatid exchange, as well as chromosomal aberrations in hamster ovary cells in addition to cell cycle delay in mice (Shelby et al., 1987). MIC has also been reported to be mutagenic in mammalian and bacterial cell cultures (Caspary and Myhr, 1986; Meshram and Rao, 1988); MIC has been estimated to have a 76.6% probability of being a genotoxic carcinogen, but only in tests with low specificity (Ennever and Rosenkranz, 1987). Mishra et al. (2009) examined the carcinogenic potential of MIC using cultured human lung fibroblasts and found that MIC induced an inflammatory response, resulting in extensive DNA damage and genomic instability.

26.7.1.6 Other toxic effects

MIC caused dose-dependent necrosis of rat brain cells in culture (Anderson et al., 1990); these findings show that MIC can exert its effects even in liquid media. Exposure of mice to 1−3 ppm MIC was found to inhibit erythroid precursors, pluripotent stem cells, and granulocyte-macrophage progenitor; recovery from this inhibitory effect was found within 3 weeks after 1 ppm but not after 3 ppm (Hong et al., 1987). At higher concentrations of 6−15 ppm, MIC inhibited cell cycling in bone marrow, alveolar cells, and T lymphocytes (Shelby et al., 1987); similar data were reported by others (Tice et al., 1987). MIC can inhibit bone marrow cell proliferation in mice (Meshram and Rao, 1988). Exposure of rats, mice, and guinea pigs to MIC vapor caused a dramatic body weight decrease in the first 2 days, which was followed by incomplete to complete recovery (Dodd et al., 1986; Bucher et al., 1987; Varma, 1987). The most likely cause of the rapid decrease in body weight is fluid loss, which may also explain the increase in hematocrit.

MIC also caused an increase in creatinine kinase, hemoglobin, hematocrit, reticulocytes, neutrophils, and blood PCO_2 in rats and guinea pigs (Dodd et al., 1986), as well as a decrease in blood pH and PO_2. MIC can cause hyperglycemia, lactic acidosis, and hypothermia in rats (Jeevaratnam and Vaidyanathan, 1992). Mishra et al. (1991) exposed rats to different concentrations of MIC vapor for 8 min and measured drug-metabolizing enzymes in lungs and found that aminopyrene demethylase and aniline hydroxylase activities were inhibited, but glutathione-*S*-transferase activity was increased.

26.7.2 Toxicity in humans

The human toxicity of MIC has been reviewed by several investigators (Mehta et al., 1990; Dhara and Dhara, 2002; Sriramachari, 2004). Toxicity in Bhopal consisted of minor eye ailments; throat irritation and cough; severe conjunctivitis, keratitis, acute bronchitis, and drowsiness; severe pulmonary edema; and convulsions followed by cardiorespiratory arrest (Kamat et al., 1985; Misra et al., 1987).

26.7.2.1 Acute toxicity

Nonlethal effects

Eye irritation, lacrimation, choking sensations, and difficulty in breathing were first reported by Kimmerle and Eben (1964), who exposed human volunteers to MIC vapor; the observations of these workers were confirmed by thousands of the victims of the Bhopal disaster. In addition, many of the victims lost consciousness, which some, but not all, regained (Varma and Saxena, 1986).

Fatal effects

Although the precise number of people who died after being exposed to MIC is still not known; our estimate, based on fatalities in 3270 households surveyed to determine effects on pregnancy (Varma, 1987), would suggest 6000−8000 deaths within 24−72 h after the gas leak in Bhopal. Deaths can be attributed to pulmonary edema. As mentioned earlier, deaths did not occur as quickly as is characteristic of cyanide poisoning; rather, it happened after a delay of several hours.

26.7.2.2 Subacute and chronic toxicity

Anecdotal reports suggest that the Bhopal disaster resulted in approximately 20,000 deaths over approximately 2 years. Since late deaths have been observed by several workers in animal models (various articles in EHP, 1987), it is reasonable to assume that there will be reports of late deaths in humans as well. It is very likely that severe lung damage accounted for most of these late deaths, although a contributory role of dehydration, internal hemorrhage, and other complications cannot be ruled out.

26.7.2.2.1 Pulmonary complications

Examination of 500 exposed people within 3 days of the Bhopal disaster (Sharma and Gaur, 1987) identified alveolar edema and destructive lesions in 8%. A retrospective study of 978 patients found mortality in 7.14%, breathlessness and cough in 95%, irritation and choking in the throat in 46%, and chest pain in 25% (Misra et al., 1987). Evidence of necrotizing lesions in respiratory tract, as well as radiological changes and compromise in lung function, has been documented (Gupta et al., 1988; Misra and Nag, 1988). Since the prevalence of compromise in lung function was higher in the population closer to the Union Carbide plant than in the population farther away, it is very likely that this occurred as a result of exposure to the toxic gases rather than preexisting bronchitis, tuberculosis, or emphysema. A follow-up of 113 exposed patients revealed worsening of pulmonary symptoms 2 years later, forced expiratory flow (FEF) between $FEF_{25\%}$ and $FEF_{75\%}$ of forced vital declined progressively over a 2-year period (Kamat et al., 1985, 1992; Patel et al., 1987), a 1- to 7-year period (Vijayan et al., 1989; Vijayan and Kuppurao, 1993; Vijayan and Sankaran, 1996), and a 10-year period (Acquilla et al., 1996; Dhara et al., 2002). Likewise, other researchers have found a direct relationship between pulmonary function compromise and inflammatory alveolitis and the severity of exposure (Vijayan et al., 1989). A causative relationship between the intensity of exposure to toxic gases and a decrease in $FEF_{25\%-75\%}$ is also suggested by another follow-up study of 454 adults conducted 10 years after the disaster (Cullinan et al., 1997).

Persistent airway hyperreactivity after a single exposure to chemical irritant has been termed *reactive airways dysfunction syndrome (RADS)* (Brooks et al., 1985). There is a strong likelihood of RADS among the victims of the gas exposure in Bhopal (Nemery, 1996); however, the question of whether the exposed Bhopal population suffers from RADS has not been carefully studied.

26.7.2.2.2 Ocular toxicity

There are reports that eye irritation and some level of lacrimation was a common experience of workers at the UCIL pesticide plant. Indeed, because of these frequent episodes of eye irritation, workers initially did not suspect that something unusual was happening in the early morning of December 3, 1984. It would seem that the eyes are most sensitive to MIC toxicity since eye irritation was experienced even by people who lived quite far from the plant and seem not to have experienced pulmonary and other symptoms (Varma and Saxena, 1986). Exposure to MIC produced ocular burning, watering, pain, and photophobia (Dwivedi et al., 1985), as well as conjunctivitis and corneal opacity (Maskati, 1986). Within the first 2 weeks of the disaster, Andersson et al. (1988) found no case of blindness in a community-based survey; surprisingly, the incidence of photophobia and interpalpebral erosion were highest in areas where the death rates were lowest.

Follow-up studies up to 2 years after the incident revealed persistent eye watering, itching, redness, photophobia, burning, Bitot's spots, and even corneal opacity (Khurrum and Ahmad, 1987; Andersson et al., 1988). It is noteworthy that in a gas-exposed cohort of 232 children admitted to the Pediatric and Eye Ward of the Hamidia Hospital, respiratory and cardiac complications were not accompanied by equally serious eye injuries (Dwivedi et al., 1985). It is very likely that poor living conditions, which favor infection (especially in children), further worsened ocular toxicity (Dhara and Dhara, 2002). On the other hand, it was feared at the time of the accident that a large number of survivors might be left with severe visual impairment; fortunately, this does not seem to be the case, which does indicate that profuse watering, a toxic effect of MIUC, also had the effect of minimizing ocular toxicity.

26.7.2.2.3 Reproductive toxicity

A follow-up study of 865 pregnant women living close to the UCIL pesticide plant at the time of the Bhopal disaster found that 379 (43.8%) did not give birth to live babies (Varma, 1987). Another follow-up of 2566 pregnant women from 18,978 households also found that 23.6% of the exposed women suffered miscarriages, as compared to 5.6% of 1218 control cohorts (Bhandari et al., 1990). Kanhere et al. (1987) found that exposure to toxic gases resulted in decreased placental and fetal weights. In addition, approximately 14% of the subjects experienced increased loss of pregnancy and infant deaths within 1 month, 2 years, and 5 years after birth, compared with 2.6%−3% within the pre-accident period (Varma et al., 1990). Other effects of exposure to MIC in women include leucorrhea, suppression of lactation, pelvic inflammatory disease, and irregular menstruation (Varma and Saxena, 1986). No effect on spermatogenesis was detected within 6 months after the Bhopal disaster (Daniel et al., 1987).

26.7.2.2.4 Genotoxicity

In a study involving 43 gas-exposed women and 40 gas-exposed men 3 years after the disaster, a significant increase in chromosomal aberrations was reported; these aberrations included breaks, gaps, and dicentric rings, which were more marked in females than in males (Ghosh et al., 1990). Chromosomal aberrations

(Goswami, 1986; Ghosh et al., 1990) and cell cycle abnormalities have been identified in Bhopal victims (Deo et al., 1987).

26.7.2.2.5 Carcinogenicity

The possibility of cancer in the Bhopal population exposed to MIC was raised by journalists. Usually, multiple contacts with a carcinogen are needed to produce cancer, but a single dose of a chemical can have the same effect (Calabrese and Blain, 1999). A cancer registry was initiated by the ICMR. However, no conclusive evidence of an increase in cancer in the exposed population has been documented. Senthikumar et al. (2011) have reported a total of 1261 cancer patients in the long-term Bhopal survivors, but they do not provide information about the size of the population examined; it is, therefore, difficult to infer whether exposure to MIC increased the number of cancer cases.

26.7.2.2.6 Immunotoxicity

Saxena et al. (1988) studied 31 exposed adults and found a significant increase in abnormal lymphocytes; however, they saw no compromise in humoral and cellular immunity in exposed populations in Bhopal. Anti-MIC antibodies were detected in blood samples from gas-exposed subjects, but the clinical implications of this discovery are not clear (Karol et al., 1987).

26.7.2.2.7 Neurotoxicity and psychological effects

Soon after the Bhopal disaster, there were displays of bizarre drawings by the surviving children, almost all of whom depicted gusts of flames going upward—starkly different from the usual pictures painted by children. Many of these drawings reflected their loss of parents or other family members or friends. While these drawings are unlikely to be specific to MIC, they probably reflected the children's response to unanticipated horror. Psychological trauma was experienced by adults, which shared many similarities with that experienced by soldiers returning from combat missions. One study categorized post-disaster psychological impact into four categories: (1) posttraumatic stress disorder characterized by anxiety, restlessness, and sleep disorder; (2) pathological grief reactions expressed as suicidal tendencies and helplessness at not being able to save family members; (3) emotional reaction to physical problems imposed upon them; and (4) exacerbation of preexisting problems (Murthy and Isaac, 1987). A survey of 164 gas-exposed children 105 days after the disaster found them apprehensive and jittery (Irani and Mahashur, 1986).

Dr. Varma and Dr. Mulay encountered a volunteer at Sambhavana Trust Clinic, which is located near the now-decommissioned pesticide plant. This young man, whose parents died during the disaster, was found to be perfectly normal on many occasions, but other workers at the clinic mentioned that, from time to time, he would become very depressed. One day in 2007, he committed suicide. There are reports of other such cases, although it is never possible to causally link such tragic events with exposure to MIC. Sethi et al. (1987) reported that a large number of survivors suffered from neurological problems, including neuroses, anxiety states, and accentuation of previous psychological problems. Bharucha and Bharucha (1987) also observed neurological and neuromuscular abnormalities in both adults and children and concluded that the incidence was lower than expected following a disaster of such magnitude as occurred in Bhopal; 24 of the 47 children examined by these authors experienced coma lasting for a maximum duration of 24 h. Neurological problems have also been reported by others (e.g., Kamat et al., 1985; Gupta et al., 1988; Misra and Kalita, 1997). Raphael and Middleton (1988) have suggested that 30%–59% of those exposed to a disaster may suffer from traumatic neuroses.

26.7.2.2.8 Other toxic effects

Soon after the disaster, Bhopal was flooded with people hoping for a cure, as well as vendors selling all kinds of medicines that they claimed would provide one. Some did not know what to expect in the days following the accident and hoped that pills would safeguard against existing ailments like loss of appetite, weakness, and breathlessness, and prevent complications.

After 35 years since the Bhopal incident, it is difficult to relate many of the symptoms, such as weakness, loss of appetite, anxiety attacks, and menstrual problems to MIC. However, many subjects continue to suffer from pulmonary dysfunctions and some compromise in visual function. As mentioned earlier, most of the victims of the disaster belonged to economically disadvantaged groups and lived in poor housing with nonexistent sanitary facilities. Therefore, they already probably suffered from many chronic diseases, including bronchitis, tuberculosis, and malaria. Even so, the Bhopal disaster certainly worsened their maladies.

26.8 Treatment

By their very nature, disasters involving chemicals pose serious problems because they concern a large number of people in a state of panic, all at the same time. Most places are not equipped to deal with such situations, especially if there is confusion about the nature of the chemical. If the chemical is a pulmonary irritant, as was the case with MIC, there is a good likelihood of suspecting cyanide poisoning, as happened in Bhopal. The other

reason for mass confusion is the erroneous belief that antidotes exist for every poison.

Laypeople are not expected to know that supportive therapy is the cornerstone of managing drug overdose or poisoning, rather than antidotes; a few exceptions include cyanide, narcotic analgesics, acetaminophen, methanol, organophosphates, digitalis, and carbon monoxide. It was legitimate for the media and people in Bhopal to demand an antidote. Therefore, conveying accurate information to the panicked population was vital. In the case of Bhopal, nondisclosure of the nature of the chemical for some period of time, inaccurate information on the cause (like the mistaken belief that it was cyanide), and lack of proper treatment proved as harmful as the poison itself. For example, if people had been warned not to run away, but instead encouraged to stay still and cover their faces with wet cloth, the benefits would have been significant. In the midst of all the confusion, the doctors in Bhopal worked out as rational a treatment as possible, which comprised of atropine, antibiotic eyedrops, and antispasmodics. Treatment of pulmonary edema requires hospitalization and positive pressure respiration; Bhopal neither had enough beds nor equipment to provide this.

Long-term treatment is also supportive and is unrelated to the initiating factors. For example, the treatment of pulmonary, ophthalmic, or neurological complications has nothing to do with whether these occur due to MIC or phosgene poisoning or due to some other cause. Cyanide, unless ingested on a regular basis from the environment or food, does not produce long-term disability if it fails to cause death.

A redeeming feature of the Bhopal tragedy was the overwhelming response of the Indian people. Hundreds flocked into Bhopal on the morning of December 3 from nearby villages, and some came from far away. In addition, doctors did a commendable job working for long hours without a break. In contrast, the medical team dispatched by Union Carbide arrived 10 days later and tried to assure the public that MIC would be destroyed rapidly and that no long-term effects were to be expected. The government of India could not summon a high-level medical team to deal with the disaster and failed miserably in following up as well. Voluntary groups and nongovernmental organizations shared a major burden of reassuring people and helping with both treatment and rehabilitation.

26.9 Toxic potential of methyl isocyanate beyond the Bhopal disaster

The Bhopal incident was the first case of mass exposure of humans, animals, and vegetation to MIC. Several factors influenced the toxicity of MIC, such as the living conditions of the victims. Under identical conditions in a developed country, the consequences most likely would have been different. At the same time, the exposure of the Bhopal population was to a specific concentration of MIC and for a specific duration.

The accidental release of MIC can happen wherever the chemical is stored. A minor leak occurred from the Union Carbide plant in West Virginia only a few months after the Bhopal disaster, and a school in the area had to be evacuated. Exposures to MIC at higher concentrations and for longer durations than in Bhopal can also occur, and these can be fatal to a substantially greater percentage of population regardless of where they happen. The fact that MIC is heavier than air makes it a potentially highly hazardous chemical, both in enclosed and open spaces.

If animal experiments were solely directed to answer questions relating to the Bhopal disaster, the use of excessively high concentrations of MIC (Dodd et al., 1986; Fowler et al., 1987) and repeated exposures would not have been very relevant. However, workers are likely to encounter repeated exposure to MIC; indeed, anecdotal reports suggest that the impending disaster was not suspected because workers were used to minor leaks (and consequently, eye irritation) in the Union Carbide plant.

No workers died inside the plant in Bhopal because MIC spewed outside the factory; however, an accident worse than Bhopal cannot be ruled out, especially if the space is enclosed.

The confusion caused by the lack of transparency about the identity of the poison by the concerned authorities at the Union Carbide headquarters and conflicting instructions only worsened the tragedy.

26.10 Benzyl chlorines and other chemicals at Bhopal

MIC and many other chemicals were stored in Bhopal. The disaster in 1984 led to the closure of the UCIL factory. While the remaining MIC was disposed of during Operation Faith, Union Carbide closed its Bhopal location, and several hundred metric tons of hazardous waste stored in open areas and sheds were not destroyed. The first study on this topic, by Dikshith et al. (1990), demonstrated the presence of 1-napathol in the soil and water in Bhopal. In all, 15 other studies were conducted between 1990 and 2010 by groups like Greenpeace International, Centre for Science and Environment (CSE), and National Environmental Engineering Research Institute (NEERI), to address the issues of whether the stored pesticides and chemicals on the factory grounds, solar evaporation ponds, and soil surrounding the factory contaminated the drinking water, and if so, which chemicals were present in levels that exceeded permissible levels. There is close agreement about the extent and type of contamination

among several studies. In studies by CSE (Johnson et al., 2009; CSE, 2013), the maximum concentration found in soil samples from specific locations like the storage, processing, and dump areas recorded contamination with carbaryl (51,003 ppm), aldicarb (7876 ppm), dichlorobenzene (2049 ppm), HCH isomers (99,700 ppm), and α-naphthol (9914 ppm), as well as heavy metals like mercury (128,000 ppm), lead (406 ppm), and chromium (1065 ppm). The NEERI report downplayed contamination of groundwater, stating that it most likely occurred due to runoff from the material dumped in the pesticide plant; but the CSE report (2013), along with several other studies, concluded that the groundwater was contaminated significantly with chemicals. All the reports acknowledged that there was very little information on the contaminants in the solar evaporation ponds where factory waste was discharged during the entire operation of the UCIL plant (CSE, 2013). Both the CSE and the NEERI reports documented the type and amount of chemicals dumped within the factory premises from 1969 to 1984. What is most surprising is that none of these reports were published in mainstream journals.

Why remediation of the site has not occurred is no secret. The cost of incineration of over 1 million metric tons of contaminated soil would be well over 10,000 million rupees, and no one—neither the Indian government nor Dow Chemical, which bought Union Carbide in 2001—was prepared to pay the bill. So, to date, very little has been done to get rid of the contaminated soil, let alone treat the contaminated water. In addition, the area surrounding the old UCIL plant has grown in population (now over 40,000 people), and these people have been drinking the chemical-laced water for 5–10 years, or even longer (up to 2 decades). There is a concern that these chemicals (some known to be teratogens) might increase the incidence of birth defects. Indeed, a systematic study by the authors is under way to document the effect on people of exposure to contaminated water, gas, or both, as compared to people who were not exposed to any toxic substances.

26.11 Concluding remarks and future directions

This chapter described the chemistry, pharmacology, and toxicology of MIC in the context of the more commonly used diisocyanates. Combatants and civilians have been subjected to lethal and debilitating chemical agents during war. However, no poisonous chemical other than MIC has the dubious distinction of killing nearly 8000 people within 72 h, and many more in the subsequent years and maiming civil society on a scale as large as what happened in Bhopal in peacetime. Unlike the September 11, 2001, terrorist attack in the United States, where the total number of casualties is accurately known, no one is certain of the exact death toll from the Bhopal incident, whether immediately afterward or in the subsequent months and years; unfortunately, the same can be said about most disasters in the developing parts of the world. While acute deaths were most likely caused by pulmonary edema, only a well-planned epidemiological study coordinated by official agencies could have determined the nature and magnitude of long-term effects. Unfortunately, such a study was not done, and many of the long-term effects cannot be identified retroactively. Carcinogenicity and genotoxicity require long-term follow-up of a large population. So far, the results have been disappointing.

MIC toxicity demonstrates that the full dimension of the pharmacology of a chemical cannot be predicted from its chemical structure, but it can be approximated by careful and painstaking research. Such an inquiry into MIC would be advisable.

Acknowledgments

This chapter is dedicated to the victims of the 1984 Bhopal disaster.

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Chapter 27

Other toxic chemicals as potential chemical warfare agents

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27.1 Introduction

For the development of new chemical weapons (CWs), a number of criteria are necessary: a research base including scientists and equipment, access to information, chemical and arms industries, and, of course, financial support. It is noteworthy that the development of CWs is possible not only for states, but also for terrorists. It is necessary to stress that the intention of this chapter is not to describe new CWs or chemical warfare agents (CWAs), but to comment on a number of trends in toxicology with the aim that these chemicals may be proposed for inclusion in the Chemical Weapons Convention (CWC) verification mechanisms. However, the text of the CWC is comprehensive and covers practically all chemicals that may be misused as CWs.

The objective of this chapter is to briefly describe a number of chemicals that could be used as toxic compounds or CWAs against humans.

27.2 General

27.2.1 Chemical weapons convention: article II, definitions and criteria

1. "CWs" means the following, together or separately:
   a. Toxic chemicals and their precursors, except where intended for purposes not prohibited under this Convention, as long as the types and quantities are consistent with such purposes.
   b. Munitions and devices, specifically designed to cause death or other harm through the toxic properties of those toxic chemicals specified in subparagraph (a), which would be released as a result of the employment of such munitions and devices.
   c. Any equipment specifically designed for use directly in connection with the employment of munitions and devices specified in subparagraph (b).
2. "Toxic chemical" means:

Any chemical which, through its chemical action on life processes, can cause death, temporary incapacitation or permanent harm to humans or animals. This includes all such chemicals, regardless of their origin or their method of production, and regardless of whether they are produced in facilities, in munitions, or elsewhere. Therefore, CWAs can be characterized as toxic chemicals. Initially, it is difficult to differentiate between the research aimed at protection against CWAs (defense) and development of new CWAs (offense). Both actions deal with the synthesis of chemicals, based on either information or ideas, or incidentally synthesized toxic compounds. A typical example is the synthesis of organophosphates (OPs) by G. Schrader, originally dedicated to the development of new pesticides. Another example of the development of a new CW would be the synthesis of OP compounds of the V series. After synthesis, the compound in question will be characterized chemically and biologically, and sometimes modified to increase its military properties (toxicity and physicochemical properties, such as stability and volatility). Then the compound can be studied in detail for its pharmacological and toxicological characteristics by using more convenient species and routes of administration. At this stage, it is practically impossible to decide if the research is offensive or defensive though some indications would lead to the opinion that the direction is offensive; for example, when attention is given to its efficacy following percutaneous or inhalation administration. Studied methods of

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dispersion under field conditions are an indication of an offensive approach; testing for protective qualities under real conditions can be regarded as a defensive approach. However, further steps, like production of large quantities and weaponization, are clearly offensive. It is useful to compare the time from synthesis to production or use of certain CWs. After the synthesis of phosgene and diphosgene (1812 and 1887), their use in 1916 was observed; a similar situation was observed for mustard (1866–1917); for CS, this period was shortened (synthesis in 1928 and use in 1950); and VX was synthesized in the early 1960s and weaponized in 1968. The big question is: What is meant by large quantities? This can be addressed using the approach contained in the CWC. Quantities would also be compared with the contamination density prescribed for different CWAs. For instance, for yperite, it is 19 tons/km^2 for percutaneous administration and 4 tons/km^2; for *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX) (percutaneous) it is 2 tons/km^2; for sarin and 3-quinuclidinyl benzilate (BZ) (by inhalation), this value is about 0.5–0.6 tons/km^2 (Robinson, 1985). Of course, it does not apply to the synthesis or production of these substances for terroristic purposes.

Apart from this "classic" approach, it would be possible to "improve" the properties of known CWs (e.g., microencapsulation so that less stable or highly volatile substances can be used). Nanotechnology offers new possibilities, as described recently by Price and Petersen (2008). The other option is to improve penetration using known enhancers like dimethyl sulfoxide (DMSO). While the percutaneous toxicity (expressed as LD_{50} in rats) of one of the toxic OPs (namely, *O*-isopropyl *S*-2-diisopropylaminoethyl methyl phosphothiolate) is 59.1 μg/kg, when it is mixed with DMSO, this value decreases to 10.1 μg/kg (Bajgar, 1989).

Binary technologies are also acknowledged; however, the development of other methods of synthesis is not excluded (the more steps involved in synthesis, the more difficult it is to control the process). An alternative is to search for compounds either used or synthesized already. From the groups of highly toxic chemicals, these could be fluorophosphorylcholines (unstable) or toxic silatrans. In the group of medications, there are also highly toxic chemicals like cardiac glycosides (digoxin), sympathomimetics (noradrenalin), and myorelaxans (succinylcholine, curare derivatives). The other compounds to be included are insulin, cantharidin, aconitin, galantamine, pancuronium, pipecuronium, some derivatives of vitamin D (cholecalcipherol), some antibiotics, cytostatics, etc. It is necessary to point out that their use is limited (e.g., parenteral administration of insulin or delayed acute effect of cytostatics). A possible candidate would be centrally acting alpha 2-adrenergics with antihypertensive and sedative properties. All bioregulators are of great interest, especially in connection with the increased possibility of obtaining significantly sufficient quantities for military and terroristic purposes. All these examples are more or less hypothetical and require further testing. There exist some groups of compounds whose misuse is more probable and some of these chemicals are under suspicion (not proved) of being introduced into military arsenals. An illustrative but not exhaustive list of warfare agents is given next.

27.3 Specific agents

27.3.1 Carbamates

Compounds in the carbamate group have a broad spectrum of toxicities—from relatively slightly toxic (carbaryl) to highly toxic compounds comparable with nerve agents (T-1123) (Fig. 27.1). As described by Robinson (1971), carbamates including T-1123 had been studied by British and Canadians as CW agents since 1940. Other carbamates were described in detail by Badawi and Hassan (1995). They are well absorbed by the lungs, gastrointestinal tract, and the skin. The clinical representation of poisoning is similar to that for nerve agents, though perhaps with more expressed peripheral signs because of quaternary nitrogen in the molecule (T-1123); therefore, penetration through the blood–brain barrier is difficult

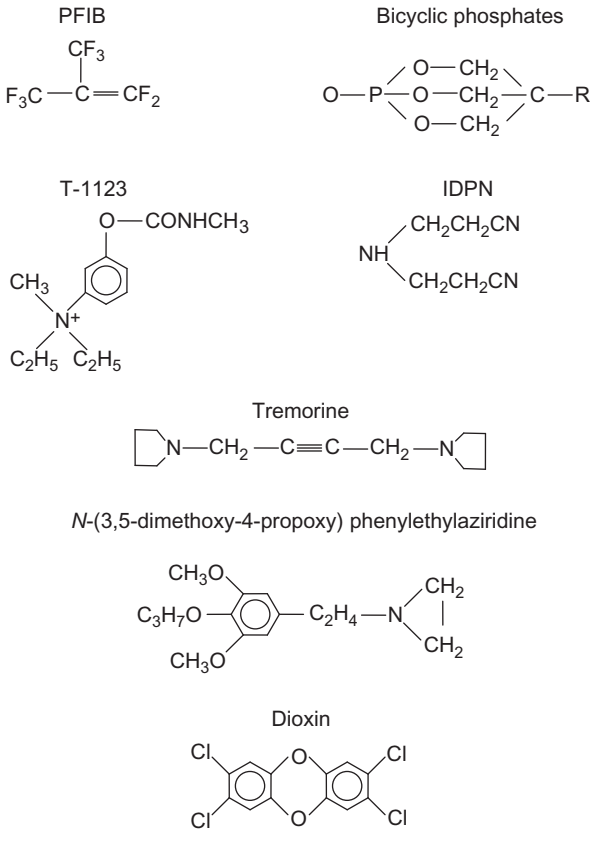


FIGURE 27.1 Chemical formulas of some toxic chemicals.

(Bajgar and Patocka, 1976; Fusek et al., 1996a). The basic mechanism of action is reversible inhibition of cholinesterases. However, the inhibition of carbamates is based on carbamylation of the active center of the acetylcholinesterase (AChE). Spontaneous decarbamylation is a relatively quick process (taking approximately 24 h) and carbamylated cholinesterases are resistant to the effect of reactivators. Therefore, the treatment is symptomatic, preferably using only atropine. These difficulties would be reasons for military use (Patočka, 1990).

27.3.2 Dioxin

Dioxin is one of the most toxic low-molecular-weight compounds (Fig. 27.1). Its oral LD_{50} for guinea pigs is 2−20 μg/kg; for monkeys, it is 2 μg/kg; for rats, it is 18−60 μg/kg; and for humans (subcutaneous administration), it is about 107 μg/kg (Bajgar, 2006; Patočka, 2004). Dioxin interferes with the metabolism of porphyrins (and the main symptom of poisoning is derived from an increase of porphyrins in the organism—i.e., porphyria cutanea tarda) by induction of delta aminolevulate synthetase. Dioxin also has carcinogenic, hepatotoxic, nephrotoxic, teratogenic, and embryotoxic effects and causes dermal changes (except porphyria cutanea tarda), including chloracne, followed by development of cachexia. There is no specific antidote, making treatment very difficult and symptomatic. Effects of dioxin following acute administration are relatively delayed. This is a limiting factor for its use as a CW. Dioxin in the organism is bound to lipids and concentration of dioxin in plasma fat in persons exposed to dioxin was 100−1000× higher than that of the normal population (Neuberger et al., 1999; Pelclová et al., 2011; Klement et al., 2013). It should be mentioned that dioxin is one of the polychlorinated biphenyls and dibezofurans that appears to be problematic for the environment (Sofronov et al., 2001; Bajgar, 2006). Dioxin was used to poison Ukraine's President Viktor A. Yushchenko (Sorg et al., 2009).

27.3.3 Bicyclic phosphates

Bicyclic phosphates have been used as flame retardants, antioxidants, stabilizers, and for spectroscopic studies. At present, however, they are being replaced by other compounds that are not as highly toxic. In a chemical structure (Fig. 27.1), when R is substituted by isopropyl, the toxicity is very close to that of sarin (LD_{50} = 0.18 mg/kg, i.m. in rats). Bicyclic phosphates act rapidly—within minutes following parenteral administration. Clinical symptoms include behavioral perturbation, muscle weakness, hyperactivity, muscle tremors, and convulsions leading to paralysis. Intoxication is slightly similar to poisoning with nerve agents, but with a different mechanism of action; that is, probably connected with gamma-aminobutyric acid receptors. Specific antidotal therapy does not exist, but a relatively good effect was observed following administration of benzodiazepines (Patočka, 2004; Bajgar, 2006).

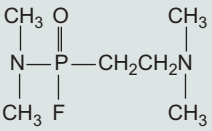
27.3.4 Perfluoroisobutene

Perfluoroisobutene (PFIB) is designated a chemical 2 by the CWC and therefore is contained in Schedule 2A. Its chemical structure is shown in Fig. 27.1. PFIB is produced by thermal decomposition of Teflon and has high inhalation toxicity characterized by pulmonary edema. PFIB has been characterized in more detail in an *ASA Newsletter* (Patočka and Bajgar, 1998). Therapy is symptomatic.

27.3.5 Organophosphates

There are other known OPs having relatively high toxicity, like amiton (Tetram), Armin, dimefox (Hanane, Terrasytam), paraoxon (E 600), and TEPP (Tetron). These compounds could be used for military and terrorist purposes; however, it would be uneconomic for the military to replace these substances. However, a new group of OP compounds has been described and characterized. This class of OPs can be described in general as 2-dialkylaminoalkyl-(dialkylamido)-fluorophosphates. In their chemical formulas, structural similarities with the group of so-called G-compounds (i.e., sarin, soman, and tabun) and V-compounds (i.e., VX and others) are found. These chemicals were designated as *GP* or *GV compounds* (Bajgar, 1992; Bajgar et al., 1992; Halámek et al., 1995; Fusek et al., 1996b). The toxicities of the most toxic derivatives are shown in Table 27.1. Intoxication with this

TABLE 27.1 LD_{50} values of GV in mice and rats following various routes of administration.

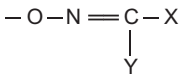


LD_{50} (μg/kg) with their 95% confidence limits

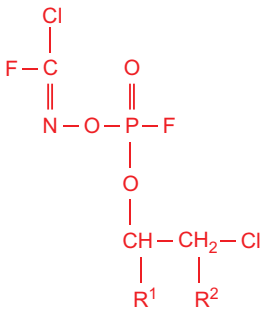
Route of administration	Mice	Rats
i.v.	27.6 (25.6−29.4)	11 (8.5−17.6)
i.m.	30.5 (28−55)	17 (15.5−23.6)
s.c.	32 (29−53)	21 (18−26)
p.o.	222 (194−255)	190 (881−272)
p.c.	Not tested	1366 (881−3138)

compound has practically the same syndromes as observed with nerve agents. Treatment with atropine and reactivators is difficult because of the absence of the ability to reactivate inhibited cholinesterases by common oximes (Kassa, 1995; Fusek et al., 1996b; Kassa et al., 2006; Kuca et al., 2006). The lack of reactivation is different from that observed for soman (i.e., aging and dealkylation) and it is probably caused by steric hindrance in the cavity of cholinesterase. The volatility of GV compounds is between VX and sarin and therefore these agents are effective when penetrating through uniforms. This is an example of an intermediate volatility agent.

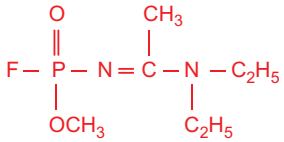
There are sources of information that suggest a new nerve agent known as Novichok or Novichok 5. In 1982, the Soviets began a secret CW development program that was code-named Foliant. The program had the apparent goal of developing new binary nerve agent weapons. Novichok has been described as a new toxic agent, and it is very difficult to treat poisoning by it (practically impossible; the toxicity was about 10 times greater than VX agents). Its exact chemical structure is unknown. The Novichok class of chemicals almost certainly belongs to the organophosphorus compounds containing the dihaloformamide group (Bajgar, 2006):



where X and Y are Cl, F, Br, or even a stable pseudohalogen like −CN. An example of Novichok (Kuca et al., 2013) could be as follows:



Another possibility could be compound A232:



There are other analogs of A232, including A230 and A234. Because these chemicals are relatively unstable, binary recepture was probably developed (Halamek and Kobliha, 2011). Novichok derivatives are close to the V agents family, and are effective at percutaneous administration. Different parts of the V agent molecules are responsible for toxicity and the rate of penetration into the organism (e.g., Bajgar et al., 2019).

27.3.6 Toxins

Toxins are prohibited by the Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction (known for short as the Biological Weapons Convention), signed in London, Moscow, and Washington on April 10, 1972. Their isolation from natural sources is sometimes difficult, but some toxins are possible to synthesize using biotechnology. Their toxicity is very high (e.g., the inhalation LD_{50} of botulinum toxin and tetanotoxin is measured in tens of $mg/kg/m^3$). Some other known toxins are saxitoxin, tetrodotoxin, and batrachotoxin. They also are highly effective by other routes of administration. Effects are observed within 10−20 min, beginning with muscle weakness, insensitivity of tongue, fingers, and mouth, followed by muscle paralysis, including respiratory muscles. Specific therapy is unknown, therefore it is necessary to save basic life functions (using artificial ventilation, etc.). When death is not observed within the first 24 h after exposure, the prognosis is relatively good.

Other types of toxins are mycotoxins, mostly trichothecenes. Their production is straightforward by fermentation, and they are highly stable and therefore may be stored for a long time. They are effective by all routes of administration, including inhalation and percutaneous absorption. Their toxicity is not very high, and the effect is prolonged. Symptoms of intoxication vary greatly, including fever, hemorrhagic eruption, bleeding, necrotic angina, decrease of leukocytes, and sepsis. Some toxins are carcinogenic, teratogenic, mutagenic, and hepatotoxic, while some also have a neurotoxic effect. There is no specific therapy; treatment is focused only on relieving symptoms (Patočka, 2004). They caused an epidemic (alimentary toxic aleukia) in the former Soviet Union and were the subject of discussion for possible use in Asia (yellow rain).

In August 1981, based on limited physical evidence, the United States announced that trichothecene mycotoxins had been used—but the findings were less than convincing to the scientific community and the issue became extremely contentious. This controversy was never totally resolved, and no definitive evidence was found (Bajgar, 2006).

The question of neurotoxins and neurotoxicity mechanisms was extensively described by Segura-Aguilar and Kostrzewa (2006). Some of these are described next.

27.3.6.1 Aziridines

Aziridines are 2-(trisubstituted phenyl) ethyl aziridines that induce changes in behavior and motor-influencing neurotransmission. Their toxicity is not very high, and they play a role in modeling a number of diseases. Their effect is long-lasting, and mostly irreversible without specific antidotal treatment. Some aziridines, for example, *N*-(3,5-dimethoxy-4-propoxy) phenylethylaziridinium (Fig. 27.1), have convulsive properties. Convulsions are treatable with benzodiazepines (Herink, 1977, 1995).

27.3.6.2 Tremorine

A relatively simple compound (Fig. 27.1), tremorine is known to induce symptoms similar to Parkinson's disease in mice and monkeys. The onset of symptoms, such as salivation, miosis, lacrimation, muscle weakness, and bradycardia, was evident within 15−30 min after administration. Typical symptoms are muscle twitch or fine tremor of the head and extremities, decrease in body temperature, and analgesia. This stage usually lasts for a few hours. Therapy is symptomatic only and not very effective (Bajgar, 2006; Patočka, 2004).

27.3.6.3 Imino-β,β-dipropionitrile

Imino-β,β-dipropionitrile (IDPN) is one of the compounds (Fig. 27.1) isolated from *Lathyrus sativus*, also called *lathyrogenic substances.* The toxicity of IDPN (and also of aziridines and tremorine) expressed as LD_{50}, is not very high, falling in the range of tens of mg/kg. Following administration of lower doses of IDPN, a condition called *waltzing syndrome* is characterized by a circling movement in both directions, and sometimes movement of the head similar to chorea (and hyperkinetic syndrome). High doses of IDPN are known to produce conjunctivitis and edema of the eyelids. In severe cases, it causes hemorrhages in the retina with the possibility of blindness. Hyperkinetic syndrome is irreversible and does not react to therapy (Bajgar, 2006).

27.3.7 Bioregulators

A variety of agents have the potential to be used as weapons of bioterrorism. These weapons have been used in wars from the start of recorded history (Metcalfe, 2002). The development of technologies on a modern militarily significant scale was initiated in several countries during the period between the two world wars (Roffey et al., 2002). However, as a result of modern technology, the risks are greater now, and the outcomes more serious (Henderson, 1999). Today, agents include not only toxins, but also a new group of compounds and bioregulators. Bioregulators are naturally occurring organic compounds that regulate diverse cellular processes in all organisms. There are substances normally found in the body that regulate normal and critical biological processes, such as blood pressure, heart rate, breathing, muscle contraction, temperature, mood control, consciousness, sleep, emotions, and immune responses. Their characteristics include activity in extremely low doses and they frequently have rapid effects. Unlike traditional disease-causing biowarfare agents that take hours and days to act, bioregulators can act within minutes after administration. There is comprehensive knowledge available on these compounds because all these compounds work as regulators and modulators of all vital biochemical pathways, linked with physiological functions of living organisms. If bioregulators were exploited for the purpose of terrorism, they could potentially cause profound pathophysiological effects. The main group of bioregulators under discussion includes different biochemicals such as neurotransmitters, hormones, and proteolytic enzymes. The common property of all bioregulators is their ability to induce biological effects and a consequential rapid fall in their concentrations in tissues. The problem is their route of administration (Patočka and Merka, 2004; Patočka et al., 2013).

A brief description of some bioregulators from a military viewpoint is given next. These peptides have been chosen based on the criteria of bioregulators intended for terrorism and warfare agents (Bokan et al., 2002).

27.3.7.1 Angiotensins

Angiotensins regulate blood pressure and contribute to sustaining hypertension (Mazzolai et al., 1998). The principal effect of angiotensin is to stimulate the synthesis of aldosterone and elevate blood pressure via vasoconstriction of the smooth muscle in arterioles.

27.3.7.2 Bombesin

Bombesin is a tetradecapeptide isolated from the skin of the amphibian frog *Bombina bombina* (Anastasi et al., 1971). It has been proposed that bombesin-related peptides may be released from the gastrointestinal tract in response to ingested food, and that they bridge the gut and brain via neurocrine means to inhibit further food intake (Merali et al., 1999).

27.3.7.3 Bradykinin

Bradykinin is a vasoactive nonapeptide, which is the most important mediator generated by the kinin system, and it is involved in inflammation processes (Calixto et al., 2000). Kinins identified thus far include bradykinin and kallidin. They cause local increases in the permeability of small blood vessels. Bradykinin is a potent stimulator of pain receptors in the skin and has a powerful influence on stimulating smooth muscle contraction, inducing

hypotension, and increasing blood flow and permeability of capillaries (Cyr et al., 2001).

27.3.7.4 Endorphins

Endorphins are peptides that bind to the neuroreceptors in the brain to give relief from pain (Terenius, 1992). Beta-endorphin is the most active, and is about 20 times more potent than morphine.

27.3.7.5 Endothelins

Endothelins constitute a family of peptides (Hart and Hart, 1992). They are very potent endogenous vasoconstrictors and vasopressors and are secreted by various cells and tissues in the human body. Of the three isoforms, endothelin-1 (ET-1) is one of the most potent contractors of vascular smooth muscles (Miller et al., 1993). Endothelins have very similar structure and biological properties to sarafotoxins (Kloog and Sokolovsky, 1989), and the toxic peptides are obtained from the venom of mole vipers (Atractaspidae).

27.3.7.6 Enkephalins

Enkephalins are endogenous pentapeptides. Two enkephalins have been identified: Met-enkephalin and Leu-enkephalin. Both enkephalins are relatively weak analgesics, which activate all opioid receptors but appear to have the highest affinity for the delta-receptors. In the central nervous system, enkephalins have been found in many areas, but predominantly those associated with nociception (Przewlocki and Przewlocka, 2001).

27.3.7.7 Histamine-releasing factor

Histamine-releasing factor (HRF) is one of the many immune system protein molecules called *cytokines*, which trigger allergic reactions. Unlike other cytokines, HRF stimulates basophils to release histamine (MacDonald, 1996).

27.3.7.8 Neuropeptide Y

Neuropeptide Y (NPY) is the most abundant neuropeptide in the brain. Its concentration is many times higher than other neuropeptides. It is a member of a family of proteins that include pancreatic polypeptide, peptide YY, and seminalplasmin. In addition to its function of stimulating feeding behavior, several other physiologic roles have been assigned to NPY, including involvement in circadian rhythms, sexual function, anxiety responses, and vascular resistance (DiBona and Neuropeptide, 2002; Halford and Blundell, 2000).

27.3.7.9 Neurotensin

Neurotensin is an endogenous peptide neurotransmitter inducing a variety of effects, including analgesia, hypothermia, and increased locomotor activity. It is also involved in regulation of dopamine pathways. Neurotensin is found in endocrine cells of the small intestine, where it leads to secretion and smooth muscle contraction (Moore and Black, 1991).

27.3.7.10 Oxytocin

Oxytocin is a nine-amino-acid peptide that is synthesized in hypothalamic neurons and transported down through axons of the posterior pituitary for secretion into blood. Oxytocin has three major physiological effects: stimulation of milk ejection, stimulation of uterine smooth muscle contraction at birth, and establishment of maternal behavior.

27.3.7.11 Somatostatin

Somatostatin is a cyclic tetradecapeptide hormone, characterized as the major physiological inhibitor of growth hormone released from the pituitary, but inhibits the release of many other physiologically important compounds, including insulin, glucagon, gastrin, and secretin (Wolkowitz, 1994).

27.3.7.12 Substance P

Substance P is an 11-amino-acid polypeptide, and a physiologically significant (and the best-known) member of a family of three related peptides known as *neurokinins*. The specific receptor subtypes corresponding to these three neurokinins are known (Sandberg et al., 1982). These neurotransmitters appear to play a key role in the regulation of emotions, and antagonists of their receptors may be the novel psychotropic drugs of the future. Koch et al. (1999) demonstrated that substance P, in combination with thiorphan, administered as an aerosol, is highly potent and extremely toxic. Exposure to the substance at extremely low air concentrations may result in incapacitation of humans.

27.3.7.13 Vasopressin

Vasopressin, also called *antidiuretic hormone (ADH)*, is a cyclic nonapeptide hormone released from the posterior pituitary. Its primary function in the body is to regulate extracellular fluid volume by affecting renal handling of water. Specific actions include inhibition of diuresis, contraction of smooth muscles, stimulation of liver glycogenesis, and modulation of adrenocorticotropic hormone release from the pituitary gland. ADH belongs to the family of vasoactive peptides involved in normal and pathological cell growth and differentiation.

27.3.8 Thyroid-stimulating hormone

Thyrotropin [also known as *thyroliberin* and *thyroid-stimulating hormone (TSH)*] is a peptide released by the anterior pituitary gland that stimulates the thyroid gland to release thyroxine (Ladram et al., 1994). The release of TSH is triggered by the action of thyrotropin-releasing factor, a peptidic substance found in the hypothalamus of the brain and influencing the secretion of glandula thyroidea.

Not long ago, most bioregulators had been unavailable in the amounts needed for terroristic attacks or military operations. However, by the end of the 20th century and beginning of the 21st century, there had been intensive developments in biomedical sciences, biotechnology, and chemical engineering in the pharmaceutical industry, and because of the revolution in the science and technology of drug discovery, the control of bioregulators will be significantly complicated.

In the near future, genomic and proteomic methods will stimulate increasing use of computer modeling techniques to identify new biologically active compounds and then determine their mode of action. Currently, new compounds are being generated in large numbers by combinatorial methods and assayed for potential activity, and it seems likely that genomic and proteomic methods will make these compounds accessible in amounts necessary for terroristic use. This is a very disagreeable situation and a new problem for the control of chemical and biological weapons.

27.4 Nonlethal weapons

Nonlethal weapons can be of a chemical, physical, or pharmacological character (Hess et al., 2005). Such weapons are designed and primarily employed for the purpose of incapacitating personnel or material while minimizing fatalities, permanent injury to personnel, and undesired damage to property and the environment (Pearson, 2006). Weapons used for physically immobilizing personnel include lasers, microwave impulses, ultrasound, electric current, nets, solidifying foams, and sliding gels. For CWs, immobilizing gases and irritants and smelly bombs can be considered. For pharmacological immobilization, ketamine, benzodiazepines, and onset accelerators are the compounds of real interest. Basic requirements for the use of immobilizing drugs are necessary, such as minimal cardiovascular and respiratory side effects; easy administration, primarily by inhalation; rapid onset; high biological accessibility and well-controlled effects; and specific antagonists that can be used within a large therapeutic range. These drugs are used for animal immobilization, using narcotizing blowpipes, darts, or guns. This group is also called *calmatives*. The compounds considered include ketamine and phencyclidine, alpha-2-agonist, opioids (including etorphine, fentanyl, and carfentanyl) and muscle relaxants (Patočka and Cabal, 2001; Dejmek, 2004; Patočka and Fusek, 2004; Streda and Patočka, 2004; Hess et al., 2005; Halamek and Kobliha, 2011; Klement et al., 2013).

As for fentanyl derivatives, there are more effective drugs than fentanyl. These include carfentanil (probably used in Moscow in 2002 against terrorists), whose effectiveness is 500−3000 times greater than morphine, and remifentanil, which is 5500−11,000 times more effective (Halamek and Kobliha, 2011). Methods of determination of fentanyl derivatives have been described (Jelinkova et al., 2013).

27.4.1 Genetic and ethnic weapons

Another possible threat is modification of the effects of commonly used chemicals or biological agents. The first administration produces no toxic effect, but the second administration of the same or another compound causes damage. The present genetic material of a human population could be misused. Recent advances in biological research could eventually lead to the creation of new types of CWs targeting a specific group of human beings with common genetic characteristics, as is perhaps the case with certain ethnic groups. Biologically, there are more similarities than differences between human beings. However, differences do exist, and if the data on ethnic differences are known, the selective effect of different chemicals to the groups cannot be excluded. Glucose-6-phosphate dehydrogenase, the enzyme that catalyzes the dehydrogenation of glucose-6-phosphate to 6-phosphogluconate, has been genetically determined and found to be low or nonexistent in some groups (though it appears more frequently in Africans and Scandinavians). It is connected with the male chromosome, and in men where it appears, hyperbilirubinemia is observed. Following administration of some normal medications, like acetylsalicylic acid, sulfadimidine, chinine, and chloramphenicol, a hemolytic syndrome is induced. Individuals with other pathologic states (e.g., chronic methemoglobinemia) will be more sensitive to drugs that are able to increase the level of methemoglobin, including analgesics, antipyretics, and nitrates. Deficiency of alpha-1-antitrypsine can cause increased sensitivity to asphyxiation agents. Plasma cholinesterase activity is also genetically determined, and individuals with decreased cholinesterase activity are more sensitive to myorelaxants. It is very probable that these people will be more sensitive to nerve agents (Bajgar, 2006).

Comparison of some criteria for the use of new chemicals for military or terroristic purposes is shown in Table 27.2.

TABLE 27.2 Different criteria for the suitability of different chemicals to be a CW.

Criterion	Carbamates	New OP	Dioxin	Bicyclic phosphates	Toxins	Incapacitants
Occurrence of first symptoms	Minutes	Minutes	Hours–days	Minutes	Different	Minutes
Death observed within	Hours	Hours	Weeks–months	Hours	Hours–weeks	Not in incapacitant doses
Availability of antidotes	Symptomatic	Yes	No	Symptomatic	Symptomatic	Yes
Easy to synthesize	+	+	+	++	+++	++
Toxicity	High	High	Very high	High	Varied	Low
Stability	+	+	+++	+	Different	++
Cost	+	+	+	++	+++	+++

For military purposes, using new nerve agents is the most likely possibility; however, their use is generally prohibited by the CWC. For terroristic purposes, there is a wide range of toxic chemicals and their choice is very dependent on the capabilities of potential users and their aims—namely, to achieve immediate effects or the liquidation or incapacitation of people over longer periods.

In the end, let us consider (very hypothetically!) terroristic use of one of the most obvious drugs: insulin. It is known that some other routes (e.g., inhalation) of administration for medical purposes have been developed (Mastrandrea, 2010). Therefore, this does not rule out the development of effective administration by inhalation. If suitable stability and aerosolization is achieved, could we realistically conclude that this drug would *not* be misused by terrorists? The effects are quick and antidotes to protect potential users would not be a problem. Military use of this drug for states or groups that are not CWC members is not ruled out either.

27.5 Concluding remarks and future directions

This chapter explains that the misuse of knowledge of pharmacology and toxicology is possible and can be applied to all human activities. The task is to be well informed in cases where it is important to know the best protection and therapeutic means, and to control all activities connected with the synthesis and possible availability of CWs/CWAs to both nonqualified and qualified people.

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Chapter 28

Ricin

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28.1 Introduction

Biological toxins are produced by a vast number of different life forms ranging from the simplest to the most complex. Each toxin has a distinctive mode of action in conjunction with a characteristic molecular structure and biochemistry. A common feature to all toxins, is that minute quantities will exert a pronounced effect on their intended targets. Algal toxins constitute a very diverse group of compounds ranging from simple ammonia to complex polypeptides and polysaccharides. Dinoflagellates are a source of some potent, nonprotein toxins, such as saxitoxin and tetrodotoxin; one such toxin causes the deleterious effects during a red tide. A red tide is the common name for an estuarial algal bloom when the photosynthetic pigments of certain species of phytoplankton can alter the hue of water; colors vary from purple to almost pink, but normally they are red or green. The mycotoxins are secondary metabolites, which are nonprotein substances produced by molds and fungi. Many molds produce more than one toxin, and in several cases, combinations of mycotoxins synergize to enhance toxicity (Gao et al., 2016; Gupta et al., 2018a,b). Leaves, roots, or seeds from some plants can be poisonous. Examples include a number of toxins produced by animals, including the nonprotein batrachotoxin found in certain species of frogs (poison dart frog), Melyridae beetles, and birds (*Pitohui*, *Ifrita kowaldi*), and a wide variety of peptides and proteins from marine snails, scorpion venoms, and snake venoms. The protein toxins ricin, derived from castor beans, and abrin, from the jequirity pea, are other examples.

In the wake of recent terrorist-related incidents across the globe, biological agents have become well-known for the threat they pose to the US and allied militaries and the civilian populations they guard. The agents are attractive to both foreign states and terrorists because they are relatively inexpensive to produce and require minimal technical infrastructure.

Ricin is defined as a biological category B agent that has the potential to pose a severe threat to public health and safety. There are currently 41 agents and toxins, including ricin, listed in the Centers for Disease Control and Prevention (CDC) regulation found in Part 73 of Title 42, Code of Federal Regulations (Possession, Use, and Transfer of Select Agents and Toxins).

Both the ricin toxin and castor bean plant have a long history of human use. The earliest human records contain references to the castor bean plant, which was known and cultivated by the ancient Egyptians. Oil pressed from the beans was used as a lubricant, and the beans were sometimes ingested to treat constipation. Even today, castor oil is still commercially produced globally. Castor oil is presently used in bath oil products, detergents, lubricants, dying agents, and most recently in the production of biodiesel fuel.

The ricin toxin is easily produced from the castor bean mash, remaining after the beans are pressed to extract castor oil (Wannemacher et al., 1992). Peter Hermann Stillmark first isolated and named the toxin ricin in 1888. Initially, the ricin protein was not known for its toxicity, but rather for causing the agglutination of erythrocytes. Later, the field of immunology was founded based on Paul Ehrlich's research with ricin. By introducing small amounts of the toxin to mice, Ehrlich was able to induce immunity specifically to ricin. This seminal work proved that in response to a challenge, certain serum proteins (antibodies) are produced that afford the host protection.

During the era of the biological weapons offensive program in the United States, ricin, by virtue of its toxicity, availability, and ease of production, was one of the first agents to be weaponized (Cookson and Nottingham, 1969). Today, ricin is one of the few biological agents utilized with known success. The most prominent of these was the attack leading to the death of the Bulgarian exile, Georgi Markov. On September 7, 1978, Markov was jabbed in the leg with an umbrella while waiting at a bus

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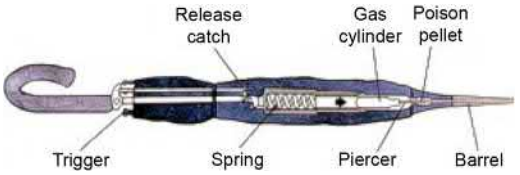

FIGURE 28.1 An artist's conception of the "umbrella gun." *From James, S.T., 2014. The Notorious Umbrella Assassination of Georgi Markov: Upon Further Investigation. <http://st-james.hubpages.com/hub/The-Notorious-Umbrella-Assassination-of-Georgi-Markov-Upon-Further-Investigation>.*

stop. A man holding the umbrella apologized to Markov before casually walking away from the scene (Crompton and Gall, 1980). Markov would later tell doctors that the man had spoken with a foreign accent. He recalled feeling a stinging pain from the site where he had been jabbed with the umbrella tip and noticed that a small, red, raised wound had formed at the site. That evening, he developed a high fever and was admitted to a hospital, where he died 3 days later. An autopsy revealed a 1.52-mm-wide metal pellet in the wound, speculated to be a watch bearing. Two 0.34-mm-diameter holes had been drilled at right angles to form a well inside the bearing. The pellet contained no trace of the poison that killed Markov, and investigators relied on standard differential diagnosis techniques and intel (military discipline that exploits a number of information collection and analysis approaches to provide guidance and direction to commanders in support of their decisions) to identify the causative agent. A host of toxins were considered, including tetanus, diphtheria, dioxin, and several nerve agents. Ricin seemed most likely because intelligence agents knew that it had been under research for decades in the Soviet Union. Working together, scientists and investigators surmised that an "umbrella gun" shot a ricin-laced pellet. The modified umbrella may have contained a cylinder of compressed air that propelled the pellet after pulling a trigger mounted in the umbrella's handle. An artist's conception of the "umbrella gun" is shown in Fig. 28.1.

Two years later, another assassination attempt with ricin occurred at a shopping mall parking lot in Tyson's Corner, Virginia, United States. Boris Korczak, a Soviet citizen who had been a CIA double agent, felt a slight sting, "something like a mosquito bite" he reported, while walking across the parking lot. He developed a fever of 106°F a few hours later, followed by a sharp decline in body temperature. Internal bleeding and an irregular heartbeat ensued. After his recovery, he extracted a tiny metal ball from his "mosquito bite" (Fig. 28.2) (Douglas and Livingston, 1987).

In another incident, ricin was discovered in a Las Vegas motel room. Roger Von Bergendorff awoke from a comatose condition on March 14, 2008, at which time he

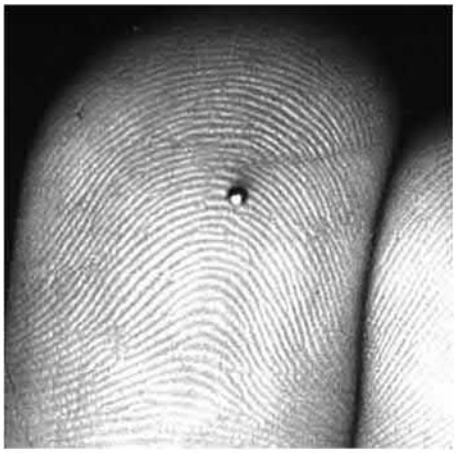

FIGURE 28.2 Ricin-laced pellet extracted from Boris Korzak.

was questioned by police as to why he had such a large quantity of ricin. Subsequently, he was arrested on April 16, 2008, and charged with possession of a biological toxin and two weapons offenses. Several other related cases involving ricin on US soil occurred in November 2003. Ricin was found in the mailroom that served Senate Majority Leader Bill Frist's office. No injuries were reported. The White House mail-processing facility and a mail-sorting facility in Greenville, South Carolina, discovered ricin. The envelope discovered in Greenville was addressed to the US Department of Transportation and labeled "Caution Ricin Poison." The enclosed letter threatened to "turn DC into a ghost town." There are more than a dozen other events that have occurred in the past (CNS, 2008).

28.2 History of biological weapons

The known use of biological weapons dates back to the beginning of written records, with many examples of their use on the battlefield. In 190 BCE, Hannibal hurled venomous snakes onto enemy ships on Pergamus at Eurymedon. Scythian archers used arrows dipped in the blood of decomposing bodies in 400 BCE. Biological weapons have also seen use in more recent times on the North American continent. In 1763, during the Pontiacs Rebellion in New England, the British officer Colonel Henry Bouquet gave blankets infected with smallpox to Native Americans at Fort Pitt, Pennsylvania, an act that devastated the Native American population. In 1863, Confederate soldiers retreating through Mississippi left dead animals in wells and ponds to deny drinking water to Union troops. Another US Civil War use of biological weapons was executed by Dr. Luke Blackburn, who attempted to infect clothing with smallpox and yellow fever for sale to unsuspecting Union troops (Diamond, 1997).

Biological weapons research and development in the United States was very active during World War I, with the impetus provided by the American Anton Dilger. After joining the German Army in 1914, Dilger experienced a nervous breakdown and returned home to the United States, which was neutral at that point. At the request of the German Government, Dilger brought strains of anthrax and glanders with him for inoculating horses awaiting shipment to Europe (Erhard, 1999). After becoming aware of Germany's involvement with biological weapons, the US Government initiated its own biological weapons program, using ricin for retaliatory use in 1918 US Department of the Army, 1977; Carter, 2000). Two methods of dispersal were examined: the adherence of ricin to pellets contained in an artillery shell and the production of a ricin dust cloud. Due to limited amounts of purified ricin on hand, and its less efficient delivery via the respiratory tract, the latter approach was considered less promising. Although both approaches were laboratory-tested to some degree, neither was perfected for battlefield use before the end of the war.

During the early 1920s, the US military leadership determined that conducting further research into the use of biological weapons was not an efficient use of resources. Germany, France, Great Britain, Canada, and Japan, however, aggressively pursued the development of offensive biological weapons (Carter, 2000). The reported use of cholera, dysentery, typhoid, plague, and anthrax by the Japanese against the Chinese motivated the United States to reinitiate a biological offensive development program. The US Army's biological warfare program began at the Edgewood Arsenal (now the Edgewood Area of the Aberdeen Proving Ground, MD) and was later moved to Camp Detrick (now Fort Detrick, Frederick, MD). The first agents produced at Camp Detrick were botulinum toxin, anthrax, and brucella, but none was weaponized before the conclusion of World War II. After the war, research continued into the weaponization of biological agents until President Nixon's decision in 1969 to redirect the research toward a defensive posture, where the United States stands today.

Because biological warfare agents are typically etiological agents of naturally occurring disease, the majority of military defensive efforts are focused on vaccine development and diagnostic testing. Although research continued, the program was only a skeleton of the former offensive effort. Despite a 1979 anthrax outbreak in Sverdlovsk, Russia, which exposed Russia's ongoing biological weapons research, the United States did not place much weight on the possibility of its forces facing biological weapons. This position, however, changed after the invasion of Kuwait by Iraqi troops and the deployment of US troops to the Persian Gulf. Iraq had a large biological agent production facility and a history of using biological and chemical agents during military offensives (Warrick, 2006). To counter this, intensive research focused on measures for protection, detection, and destruction; these efforts are still ongoing today. In addition to state-sponsored entities, the revelation that terrorist organizations, such as the Aum Shinrikyo in Japan, were actively developing biological agents has brought attention to domestic defense against biological weapons. Defending against these agents requires not only an understanding of the agent but also an understanding of how an adversary might utilize it.

28.3 The weaponization of biological agents

Although many pathogens and toxins cause disease or toxicity, relatively few of the naturally occurring agents can be adapted for use as a biological weapon. Paramount is its ability to survive the mechanical rigors of large-scale aerosol dissemination. Other important characteristics include an agent's availability, ease of production and storage, its lethality, or its ability to incapacitate through morbidity (North Atlantic Treaty Organization, 1992). While some pursue agents of death, the philosophy of others is that the most attractive agents are those that incapacitate an adversary.

During the era of the US biological offensive program, agents such as *Staphylococcus aureus* enterotoxin B (SEB) and Venezuelan equine encephalitis were selected for their ability to incapacitate rather than kill. It was estimated that an afflicted soldier would require the care from a minimum of three personnel, whereas a dead soldier placed no burden on personnel. Providing care to sickened forces can quickly render entire military units mission-ineffective by overloading field-forward medical facilities intended to treat the wounded. In addition, these same agents could also be turned on civilians, concentrating the population at care centers, which in turn would facilitate attack and invasion by our forces. A final mark of an ideal agent is one that renders the adversary defenseless while the disseminating force is unaffected.

Terrorist organizations have historically lacked technical expertise; this often leaves insurmountable challenges for large-scale production and agent delivery. Thus, these organizations require an agent that can be easily disseminated in open air using common items such as crop dusters or liquid spray devices. Agents that can survive drying have high inhalational efficiency and can effectively cause infection or toxicity, which makes them ideal biological threat agents. The options for indoor dissemination or individual targets are much broader and essentially only limited to the creativity of the perpetrator. Use of the postal service, for instance, before the anthrax attacks on the United States, was believed to be impossible.

During decontamination efforts after the anthrax attack, it was also discovered that reaerosolization was a threat. The optimal agent particle size is dependent on the delivery mode. For aerosol dissemination, the smaller the particle size, the longer it will stay airborne and the more likely it can be reaerosolized after settling. Bacterial agents have a minimum size that is limited by their cellular dimensions; this is typically 1−5 μm. Alternatively, viral agents can be as small as 0.1 μm, and toxins are limited only by their molecular weight. It has been shown that particles of less than 10 μm can stay airborne for extended periods of time (Winters and Chenoweth, 2002). Particle size is inconsequential for attack via injection, ingestion, or transdermal transmission.

Agent stability after production is another important factor to consider when selecting an agent for weaponization. Environmental factors, including temperature, relative humidity, atmospheric pollution and sunlight, can all have an effect on agent viability. Feasible options for storage of agents are as liquid samples at room temperature, as liquid samples under refrigeration, as dry samples at room temperature, and as dry samples under refrigeration. The most desirable option is dry sample storage at room temperature, because this eliminates the need for refrigeration, allows for particle sizing before dissemination, and minimizes the environmental effects on dissemination.

By all means, toxins are probably the most suitable biological agents for use in any attack. In contrast to replicating agents, using toxins as biological agents requires that a lethal amount must be injected, ingested, or inhaled. Given this caveat, a sizeable amount of toxin must be available for dispersion in large-scale attacks, severely limiting the effective toxins to those that are the most lethal and easily produced. Ricin is one of only a few toxins that meet these requirements, along with botulinum toxin, SEB, and the trichothecene mycotoxins (Haschek and Beasley, 2009). Principally, because of its suitability as a biological warfare agent, the ricin toxin has been one of the biological weapons of choice for state-sponsored organizations (Kortepeter and Parker, 1999).

28.4 The family of ribosome-inactivating proteins

Ribosome-inactivating proteins (RIPs) are toxins that act intracellularly. They consist of two different subunits: a subunit A that is responsible for the enzymatic activity of the toxin and a subunit B that binds to a specific receptor on the host cell membrane, thus allowing the A subunit, the enzyme, to cross the cell membrane (Spooner, and Lord, 2011). The enzymatic component is not active until it is released from the native (A + B) toxin. Isolated A subunits are enzymatically active but lack binding and cell entry capability. Isolated B subunits may bind to target cells (and even block the binding of the native toxin), but they are nontoxic.

There are a variety of ways that toxin subunits may be synthesized and arranged: A + B indicates that the toxin is synthesized and secreted as two separate protein subunits that interact at the target cell surface; A-B or A-5B indicates that the A and B subunits are synthesized separately, but associated by noncovalent bonds during secretion and binding to their target; 5B indicates that the binding domain of the protein is composed of five identical subunits. A/B denotes a toxin synthesized as a single polypeptide, divided into A and B domains that may be separated by proteolytic cleavage (Todar, 2014).

Ricin is a ribosome-inactivating lectin isolated from the beans of *Ricinus*. Ricin and other plant lectins, for example, abrin and modeccin, consist of two peptide chains, A, the toxin, and B, a lectin, linked together by a disulfide bond. Lectins are a class of carbohydrate-binding proteins that interact specifically with glycoconjugates present in other organisms. Specific carbohydrate-binding activity distinguishes lectins from other plant proteins (Zhu-Salzman et al., 1998). This interaction is as specific as enzyme−substrate or antigen−antibody interactions.

Another characteristic property of lectins is that they agglutinate cells and precipitate polysaccharides and glycoproteins. That is because lectins are, as a rule, divalent or oligovalent, meaning, each lectin molecule has at least two carbohydrate-binding sites that allow cross-linking between cells (by combining with sugars on their surfaces) or between sugar-containing macromolecules. Lectins have accordingly been defined as sugar-binding proteins of nonimmune origin that agglutinate cells and precipitate polysaccharides or glycoproteins (Goldstein et al., 1980).

Lectins are present in numerous edible plants. For many years it has been known that they occur in major food sources for humans and animals such as soybeans, kidney beans, lima beans, mung beans, lentils, garden peas, and peanuts. One family is composed of proteins that contain one or more 30- to 43-amino-acid cysteine-rich chitin-binding domains. Another family, mainly legume lectins, binds carbohydrate substrates by way of interactions involving specific amino acid residues that are located spatially throughout the peptide. The exact role of lectins in plants is unclear, although they can serve as potent insecticides. Castor beans contain so much lectin that they are toxic to most organisms. The binding ability and specificity of the lectin is preserved when bound to the toxic A chain. It is the lectin that directs the toxin to its target within the cell. There are a number of toxins that are bound to lectins. These are all classified as type III toxins. This classification is based on the mode of

entry into the cell. There are two categories of type III toxins: those that are bound to a single lectin, referred to as an AB toxin, and those that are bound to four lectins, known as AB5 toxins. The most commonly known type III toxins are listed here.

AB toxins (usually plant toxins):

1. Ricin;
2. Abrin;
3. Mistletoe (viscumin);
4. Modeccin;
5. Diphtheria.

AB5 toxins (usually bacterial toxins):

1. *Campylobacter jejuni* enterotoxin (from *Campylobacter jejuni*);
2. Cholera toxin (*Vibrio cholerae*);
3. Heat-labile enterotoxins (LT and LT-II) (*Escherichia coli*);
4. Pertussis toxin (*Bordetella pertussis*);
5. Shiga toxin (*Shigella dysenteriae*);
6. Shiga-like toxin (or verotoxin) enterohemorrhagic varieties of *E. coli* including (O157:H7).

Cholera toxin, Shiga toxin, ricin, and abrin are protein toxins that damage mammalian cells by a mechanism that includes four essential events: receptor-mediated endocytosis; retrograde transport into the lumen of the endoplasmic reticulum (ER); passage through the ER membrane into the cytoplasm; and catalytic inactivation of a target substrate in the cytoplasm. The receptor-binding site of a protein toxin is usually within a subunit or domain that is distinct from the subunit or domain that bears the catalytic activity of the toxin. Both cholera toxin and Shiga toxin belong to the AB5 protein toxin family in which the A chain is the enzymatic subunit, and each of the five identical B chains binds cell surface receptors.

Some peptide and protein toxins have been classified into families containing a number of different toxins with the same biological activity. Within any one family, the toxins can differ in amino acid sequence and number, but their molecular architectures and active site conformations are conserved. One of the most intensely studied toxin families is that of the RIPs, which includes ricin. The term "ribosome-inactivating protein" was introduced to designate plant proteins that inactivate animal ribosomes. The toxins were later found in certain bacteria and fungi (Endo et al., 1988). These are cytotoxins that catalytically and specifically inactivate the large subunit of eukaryotic or prokaryotic ribosomes (Stirpe and Barbieri, 1986). Historically, RIPs have been classified into two types based on their quaternary structure.

The type I RIPs are the most numerous and are all synthesized as a single-chain enzyme of approximately 30 kDa. Type II RIPs are synthesized as larger precursors that accumulate in protein bodies and contain an approximately 30-kDa A-chain linked by a disulfide bond to a lectin B-chain of similar size (Stirpe and Barbieri, 1986). Plant RIPs, including all type I toxins and the A-chains of type II toxins, are RNA *N*-glycosidases capable of hydrolyzing the nitrogen−carbon glycosidic bond of a specific adenosine located in the sarcin/ricin domain of the largest ribosomal RNA (Barbieri et al., 2006). The B-chain is able to bind the D-galactose-terminated receptors on the membranes of animal cells and facilitates the internalization of the enzymatic A-chain. To date, more than 50 type I RIPs and approximately 15 type II RIPs have been identified (Stirpe and Barbieri, 1986). Although there is some variation at the N- and C-termini of their respective polypeptide chains, the active sites of the type I RIPs and the A-chains of type II RIPs are well-conserved, as are their three-dimensional conformations (Gasperi-Campani et al., 1985).

Originally, type I and type II RIPs were identified based on biological activities. Type II RIPs were discovered more than a century ago, when Stillmark isolated the toxic protein ricin from castor beans. As mentioned, the toxicity of ricin was initially attributed to its agglutination activity for red blood cells and not by ribosome inactivation. Type II RIPs attribute their carbohydrate-binding activity to their B-chain, which contains two or possibly three binding sites (Robertus, 1991). The multiple binding sites allow B-chains to aggregate with red blood cells and platelets.

Duggar and Armstrong observed that the so-called *Phytolacca Americana* antiviral protein (PAP) inhibited the transmission of tobacco mosaic virus in plants. In 1978, PAP was recognized as an inhibitor of protein synthesis. Not all type I RIPs, however, are believed to be antiviral proteins (Lam and Ng, 2001). Unlike their type II counterparts, type I RIPs exhibit low toxicity because they are not able to bind and cross the cell membrane efficiently. Type I RIPs, however, are cytotoxic to certain cells such as macrophages. The cells can absorb type I RIPs by pinocytosis and subsequently succumb to RIP activity. Recently, a type III RIP has been isolated from *Hordeum vulgare*, the common barley plant. The RIP consists of an amino-terminal domain resembling type I RIP and is linked to an unrelated carboxyl-terminal domain with unknown function (Bolognesi et al., 2002). The type III RIP accumulates in the barley kernel as an inactive precursor that, on germination, is processed into a two-chain RIP by removal of an internal peptide from the catalytic domain (Peumans et al., 2001).

RIPs have been identified in more than 50 different plant species and have also been found in fungi, bacteria, and at least one algal species. The highest quantity of RIPs has been isolated from the carnation family Caryophyllaceae, the elder family Sambucaceae, the

FIGURE 28.3 Photograph of a castor bean plant (A) and castor beans (B). *From Audi, J., Belson, M., Patel, M., 2005. Ricin poisoning: a comprehensive review. JAMA 294, 2342–2351.*

family of gourd-producing plants Cucurbitaceae, the Euphorbiaceae family of flowering plants, the pokeweed family Phytolaccaceae, and the family of flowering grasses Poaceae (Reinbothe et al., 1994). Presently, the role of RIPs in plant physiology is not entirely clear. Based on their variable activity toward heterologous and autologous plant ribosomes, several possible roles have been proposed: antiviral activity, antifungal activity, herbivore defense, a role in the arrest of cellular metabolism during periods of senescence, and, finally, a role as storage proteins (Bolognesi et al., 2002). Conclusive evidence has been obtained that RIPs not only deadenylate ribosomal RNA but are also capable of removing adenine residues from DNA and several other polynucleotide substrates. Thus, it has been proposed to rename RIPs as polynucleotide:adenosine:glycosidases (Shakirova and Bezrukova, 2007).

28.5 The ricin toxin structure and biosynthesis

Ricin is a toxin originating from *Ricinus communis*, the castor bean plant. A photograph of the plant is shown in Fig. 28.3A; a photograph of the beans it produces is presented in Fig. 28.3B. As for all type II RIPs, ricin is a protein consisting of two folded peptide chains, designated the A-chain and B-chains. The two are linked by a single disulfide bridge. Ricin has a molecular weight of approximately 64–65 kDa; ricin A-chain (RTA) is a 267-amino-acid globular protein domain containing eight α-helices and eight β-sheets (Wright and Robertus, 1987). The catalytically active site occurs as a long cleft on the A-chain surface. Within the active site, glutamic acid 177 is a key catalytic residue, and conversion to glutimate reduces activity 180-fold.

Ricin B-chain is a 262-amino-acid protein domain that conforms to a barbell-shaped architecture. The folded domain has a sugar-binding site at each end, allowing hydrogen bonding to galactose and *N*-acetyl galactosamine typically found on cell surfaces. The B-chain is not toxic and the A-chain alone is not toxic. In fact, many

FIGURE 28.4 Representation of the ricin protein derived from X-ray crystallographic data. The A-chain and B-chain are blue and orange, respectively. Random coil regions are strings, β-sheets are flat ribbons, and α-helices are coiled ribbons. *From GS, 2014. <Globalsecurity.org> <http://www.globalsecurity.org/wmd/intro/bio_ricin-pics.htm> (accessed 29.04.14.).*

edible plants such as barley synthesize A-chain. The B-chain has been expressed in monkey COS-M6 cells with no adverse effects (Weston et al., 1994). A representation of the ricin protein based on X-ray crystallographic data is presented in Fig. 28.4.

The biosynthesis of ricin and RCA occurs in the endosperm cells of maturing *R. communis* seeds for storage in subcellular vacuolar compartments referred to as protein bodies. This entire process is illustrated schematically in Fig. 28.5. Biosynthesis begins with the translation of a propolypeptide encompassing both the A-chain and B-chain as well as an amino-terminal signal sequence (Butterworth and Lord, 1983; Lord, 1985). The signal sequence directs the nascent chain to the ER lumen, where it is cleaved and removed from the rest of the long polypeptide chain. The resulting proricin is N-glycosylated in the lumen, and protein disulfide

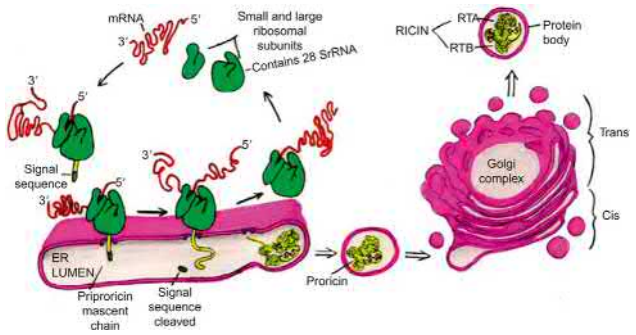

FIGURE 28.5 Biosynthesis of the ricin toxin. *From EHSO, 2014. Environment, Health and Safety Online. <http://www.ehso.com/ricin.php> (accessed 29.04.14.).*

isomerases catalyze disulfide bond formation as the proricin chain folds into two globular protein domains. Proricin undergoes further oligosaccharide modifications within the Golgi complex before it is transported within vesicles to the protein bodies (Hiraiwa et al., 1997). The toxins are stored in the protein bodies until their ultimate destruction by hydrolysis, typically only a few days after germination of the mature (Lord, 1985).

28.6 The cellular internalization of ricin

The entry of ricin into a living cell begins with the reversible binding of its B-chain to cell-surface glycolipids and glycoproteins bearing $\beta(1\rightarrow4)$-linked galactose residues (Moya et al., 1985). The toxin has two galactose-binding sites for cell surface binding, but ricin may also be bound to certain cells by an entirely different mechanism. Ricin, a glycoprotein, contains mannose-rich N-linked oligosaccharides in both of its constituent subunits. On the surface of cells in the reticuloendothelial system, mannose receptors are present that have been shown to bind these carbohydrates (Simmons et al., 1986). Further, the binding by these mannose receptors results in subsequent cell death (Simmons et al., 1986), and deglycosylation of the toxin abolishes both binding and intoxication by the mannose receptor pathway (Foxwell et al., 1987). It is estimated that 10^6-10^8 toxin molecules may bind to a single cell at one time. The toxin molecules are internalized into cells via endocytosis in coated pits and vesicles, or smooth pits and vesicles (Moya et al., 1985). Once in the cytosol, these vesicles fuse with endosomes that can return some of the toxin molecules to the cell surface by exocytosis or, as shown in Fig. 28.5, endosomes can fuse with lysosomes that will destroy the ricin molecules (Sandvig and Olsnes, 1979; Sandvig et al., 2004). Alternatively, the endosomes can carry ricin molecules to the Golgi complex and ER by retrograde transport (Sandvig et al., 2004) (Fig. 28.6). Ricin molecules reaching the trans Golgi network can penetrate its membrane and reach the cytosol

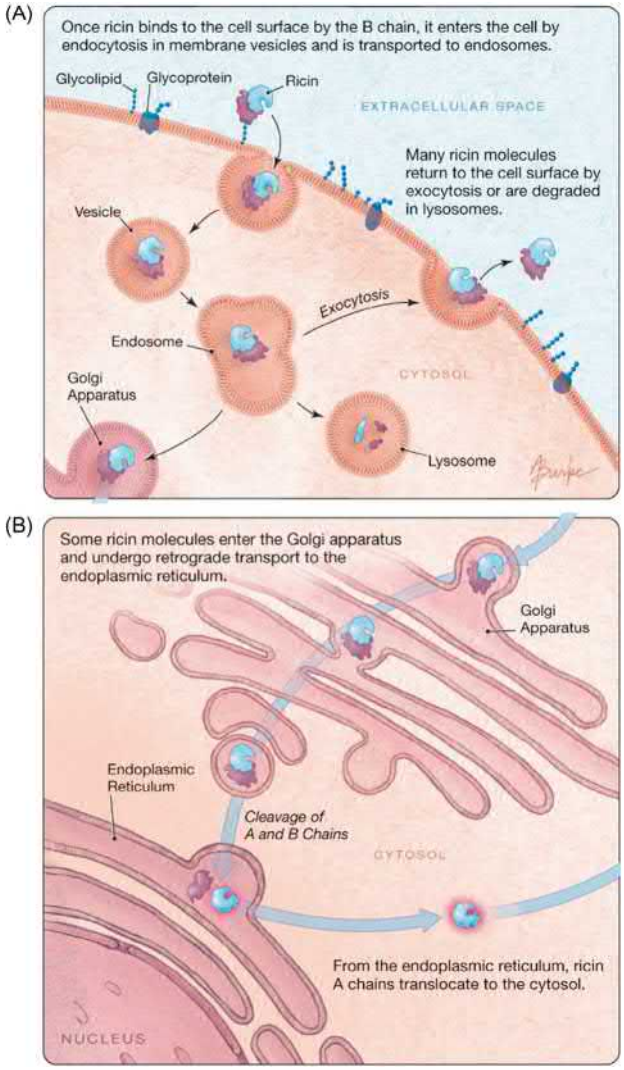

FIGURE 28.6 Example of the cellular internalization of ricin. The process involves endocytosis by coated pits and vesicles (A) or smooth pits and vesicles, followed by vesicle−endosome fusion (B). Ricin molecules can then return to the cell surface by exocytosis, or the vesicles may fuse to lysosomes for toxin destruction. *From Audi, J., Belson, M., Patel, M., 2005. Ricin poisoning: a comprehensive review. JAMA 294, 2342−2351.*

or, in some cases, return to the cell surface (Sandvig et al., 2004). For ricin molecules reaching the ER lumen, the disulfide bond connecting their A-chain and B-chain will be reduced, facilitating a partial unfolding of the A-chain (Kornfeld et al., 1991). The unraveling toxin protein will be translocated across the ER membrane via the Sec61p translocon, following the same pathway as that for incorrectly folded ER proteins targeted to the ER-associated degradation machinery (Simpson et al., 1999; Lord et al., 2003). Ricin molecules escaping the degradation machinery may find their way into the cytosol. These ricin molecules, and those crossing the trans Golgi network membrane into the cytosol, can refold into a

protease-resistant, enzymatically active structure (Lord et al., 2003). Just a single ricin molecule entering the cytosol of a living cell with a K_{cat} of 1500 min^{-1} can inactivate enough ribosomes to ultimately result in cell death (Endo et al., 1987).

28.7 *N*-Glycosidase activity of ricin

The ricin A-chain contains an enzymatically active site that recognizes and binds a highly conserved region in the large 28S rRNA of the intoxicated cell, referred to as the sarcin/ricin loop (Rajamohan et al., 2001). This loop is a very short stem-loop structure of the overall ribosome and the loop is located in domain VII, approximately 400 nucleotides from the 3′-end of the rRNA. Within the stem-loop, the ring of a single adenine (A_{4324}) becomes sandwiched between two tyrosine rings in the catalytic cleft of the ricin A-chain and is hydrolyzed at the carbon–nitrogen glycosidic bond by the *N*-glycosidase action of ricin (Endo et al., 1987). This particular site-specific RNA *N*-glycosidase activity is a common property of all identified type I and type II RIPs (Barbieri et al., 2006). The activity prevents the binding of elongation factors EF-1 and EF-2, resulting in the cessation of mRNA translation.

Although all RIPs exhibit *N*-glycosidase activity toward ribosomes, they display marked differences in substrate specificity. Most type I toxins exhibit very broad specificities, whereas type II toxins display a preference for animal ribosomes. Ricin, for example, is highly active against mammalian and yeast ribosomes but poorly active, or even inactive, against plant and bacterial ribosomes (Yoshinari et al., 1997). In contrast, pokeweed antiviral protein depurinates ribosomes from plants, bacteria, yeast, and various evolutionarily diverse animals (Rajamohan et al., 1999).

Both the RIP and ribosome in a RIP–ribosome interaction contribute to the apparent substrate specificity (Kurinov et al., 1999). Due to the universally conserved rRNA sarcin/ricin loop structure, differences in sensitivity to different RIPs may possibly reside within the associated ribosomal proteins that confer tertiary structure to the ribosome (Kurinov et al., 1999). These differences may either allow or prevent access of the RIPs to the sarcin/ricin loop. Vater et al. (1995) identified the rat liver ribosomal proteins L9 and L10e as the binding target of the ricin A-chain, whereas yeast ribosomal protein L3 has been identified as the binding factor for pokeweed antiviral protein. The specific interaction between pokeweed antiviral protein and L3 is most likely explained by the broad-spectrum activity of pokeweed antiviral protein toward ribosomes from species of different taxonomic groups, due to the highly conserved L3 in ribosomes (Rajamohan et al., 1999). Differences in activity and ribosome substrate specificity are also due to differences in the structure of different RIPs. This was demonstrated by an approach in which pokeweed antiviral protein–ricin A-chain protein hybrids were created and examined for activity on rabbit reticulocyte and *E. coli* ribosomes (Vater et al., 1995).

Experimental results demonstrated that the aminoterminal half of the hybrid proteins determines the substrate specificity. Structurally dissimilar surface polypeptide loops apparently do not play a role. In addition to the highly specific action on ribosomes, ricin and related RIPs have a less specific action in vitro on supercoiled double-stranded DNA, single-stranded DNA (ssDNA), and RNA substrates releasing multiple adenine residues and, in some instances, guanine residues (Wang and Tumer, 1999). RTA also catalyzes the hydrolysis of synthetic oligonucleotides as short as six base pairs, provided a GAGA tetra loop is present (Amukele and Schramm, 2004). Other reported RIP activities are lipase, chitinase, and superoxide dismutase (Xu et al., 2008). To date, activity against synthetic DNA or RNA oligonucleotides for the various RIPs has not been compared.

28.8 Signs and symptoms of ricin exposure

As with most biological warfare agents, ricin remains biologically active through several different modes of entry into a living animal, including ingestion, injection, and inhalation. Symptoms and toxicity, however, can vary dramatically with mode of entry (Olsnes, 2004). Response to cutaneous contact with ricin, however, is not particularly noteworthy. An urticarial, IgE-mediated allergic reaction may occur after handling the intact castor bean plant or exposure to castor bean dust or pomace. Irritation and the development of pseudomembranous conjunctivitis after ocular exposure to very low ricin concentrations are commonly reported. In humans, general signs and symptoms of ricin exposure include fever, fatigue, weakness, muscle pain, and dehydration.

Most of what is known about the effects of ingested ricin derives from animal studies, which reflect a 100-fold variation in lethal toxicity between the various animals studied. Of all tested animals, the chicken is the least sensitive and the horse is the most sensitive. In mice, the lethal dose for 50% of an animal test group (LD_{50}) is 20 mg/kg when ingested (Ishiguro et al., 1992). Absorption occurs within 2 h with uptake by both gastrointestinal lymphatic and blood vessels, and approximately 20%–45% of the ingested ricin is excreted unchanged in the feces (Ishiguro et al., 1992). The toxin accumulates mainly in the liver and spleen, with the onset of symptoms usually occurring within 4–6 h (Challoner and

McCarron, 1990; Ishiguro et al., 1992). Initial symptoms are nonspecific and may include abdominal pain, vomiting, diarrhea, heartburn, and oropharyngeal pain (Klaim and Jaeger, 1990). Without treatment, fluid losses may lead to electrolyte imbalances, dehydration, hypotension, and circulatory collapse. Blood chemistry changes can lead to leukocytosis, elevated transaminases and creatinine kinase activities, hyperbilirubinemia, renal insufficiency, and anemia. Postmortem findings of diffuse intestinal hemorrhagic lesions are common (Fodstad et al., 1976; Klaim and Jaeger, 1990).

The majority of the data for ricin injection derive from rodent studies (Beyer et al., 2009; David et al., 2009). The ricin LD_{50} in mice is approximately 5 μg/kg by injection (Fodstad et al., 1976). The highest concentration of ricin was found to localize to the spleen after either intravenous or intraperitoneal administration of ^{125}I-ricin, whereas the urine was found to be the major route of elimination. In general, for rodents, the majority of ricin excretion occurs in the urine over the course of the first 24 h (Blakey et al., 1988). The onset of nonspecific signs and symptoms, which may be similar to sepsis (fever, headache, dizziness, nausea, anorexia, hypotension, abdominal pain), occurs within 12 h after dosing. Abnormalities include elevated liver transaminases, amylase, and creatinine kinase activities, hyperbilirubinemia, myoglobinuria, and renal insufficiency, and these can progress to multisystem organ failure (Ishiguro et al., 1992; Bradberry et al., 2003). Postmortem findings are consistent in forensic case investigations as well as animal studies and include focal hemorrhage in the intestines, brain, myocardium, and pleura (Fodstad et al., 1976). The lymph nodes, kidneys, and intestines may also show signs of necrosis, hemorrhage, and edema (Flexner, 1897).

In cases of ricin inhalation, particle size can significantly influence lung deposition and lethality. Smaller particles typically deposit deeper into the respiratory tract, resulting in higher mortality. Larger particles deposit higher in the airways and can be swept up into the mouth by the mucociliary system and subsequently swallowed (Roy et al., 2003). The LD_{50} in mice exposed to ricin particles smaller than 5 μm is between 3 and 5 μg/kg (Wilhelmsen and Pitt, 1996). For monkeys, inhalation of 1- to 2-μm particles at 21–42 μg/kg results in progressive dyspnea 20–24 h after dosing (Wilhelmsen and Pitt, 1996). Postmortem findings typically include diffuse pulmonary edema with multifocal areas of necrosis and inflammation, and injury tends to be significantly worse in the distal airways and alveoli (Soler-Rodríguez et al., 1993; Griffiths et al., 1995; Wilhelmsen and Pitt, 1996; Brown and White, 1997). Toxicity results directly from the inhibition of protein synthesis, release of cytokine mediators, and direct injury to the epithelial membrane, with the primary targets of toxicity as the type I and type II pneumocytes (Soler-Rodríguez et al., 1993; Griffiths et al., 1995; Wilhelmsen and Pitt, 1996; Brown and White, 1997; Roy et al., 2003). Significant systemic absorption is usually not observed after inhalation, and toxicity is primarily limited to the respiratory tract (Soler-Rodríguez et al., 1993; Wilhelmsen and Pitt, 1996). Respiratory failure is likely to be the primary cause of morbidity and mortality in humans after ricin inhalation, and symptoms include severe lung inflammation with progressive cough, dyspnea, cyanosis, and pulmonary edema (USAMRIID, 2011).

The CDC recommends taking the following precautions when handling ricin in the laboratory (Centers for Disease Control and Prevention, 2014):

- Avoid any activity that places persons at risk for exposure, especially activities that might create aerosols or droplet dispersal.
- Follow laboratory safety practices to prevent exposure.
- Decontaminate laboratory benches after each use and dispose of supplies and equipment in proper receptacles.
- Avoid touching mucosal surfaces with gloved or ungloved hands and never eat or drink in the laboratory.
- Remove gloves before leaving the laboratory, dispose of gloves in a biohazard container, wash hands, and remove laboratory coat.

28.9 Field-forward biological agent detection

28.9.1 Immunoassays

Conventional assays used to detect biological toxins involve traditional biochemical techniques such as immunoassays based on antibodies raised against the toxin target. Immunoassays were first developed in the 1950s; 30 years later, the assays were in common use due to the development of standard reagents and automated assay readers (Ngundi et al., 2006). The immunoassay is a rapid and reliable technique for identifying a molecule that contains at least one antigenic moiety. Immunoassays rely on the inherent ability of an antibody to bind specific chemical groups on conformationally flexible antigens, such as carbohydrates, or specific molecular conformations in the case of large, conformationally rigid molecules, such as proteins. These chemical groups and conformations are referred to as antigenic determinants and epitopes, respectively. Antibodies are ideal for use in the detection of biological molecules, because they are produced against a specific determinant or epitope, which makes them highly specific and selective. Field-forward analysis for biothreat agents is limited to antibody-based tests and polymerase chain

reaction (PCR). The most commonly used tests are the ELISA and immunochromatographic tests typically referred to as hand-held assays (HHAs), as shown in Fig. 28.7.

Currently, the gold standard of immunoassays for toxin detection is the enzyme-linked immunosorbent assay (ELISA) (Hammond et al., 2006; Guglielmo-Viret and Thullier, 2007; Garber and O'Brien, 2008). In its simplest terms, the ELISA is a technique used to detect the presence of an antigen in a sample by affixing the sample contents to a solid support and visualizing the immobilized antigens with specific antibodies. Performing an ELISA involves at least one antibody with specificity for the target antigen. The sample is first immobilized to a solid support such as the surface of a polystyrene microtiter plate, either nonspecifically via direct adsorption or specifically via capture by another antibody specific for the target antigen that is affixed to the solid support. After antigen immobilization, a second mixture of antibodies sometimes referred to as detector antibodies is added to the complex with target antigens. Detector antibodies can be covalently linked to an enzyme or a chromophoric reporter group, or themselves can be detected by a secondary antibody linked to an enzyme or chromophoric reporter group. The labeling of the detector antibody allows for visualization of the antibody/antigen complex. Washing with a mild detergent solution typically is used between each step to remove nonspecific antibodies or other proteins. After a final wash, the assay is developed to visualize the results, either by addition of an enzymatic substrate to produce a color change or precipitate, or by irradiation of a specific wavelength of light in the case of chromophoric reporter groups.

The most recently developed technique aimed at enhancing immunoassay sensitivity is immune-PCR. The technique is markedly more sensitive than ELISA, primarily because it combines the detection specificity of an antibody with the amplification power of real-time PCR for a nucleic acid. The technique was first described by Sano et al. (1992). Realized limits of detection have surpassed ELISA by 10-fold to 10,000-fold. Typically, lateral flow immunochromatographic assays are used in field-forward situations due to lenient storage and equipment requirements (Pastoor et al., 2008). The problem with PCR-based assays is that they do not detect toxin protein, but they do detect copurifying DNA, which is not always present. Cell-based bioassays are sometimes used to confirm intact toxin-killing activity of the ricin in environmental samples (Brzezinski and Craft, 2007).

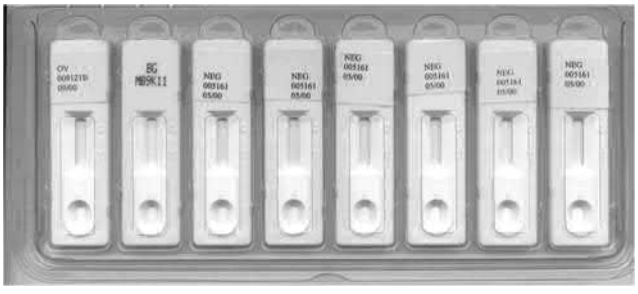

FIGURE 28.7 Photograph of an HHA packet.

The ricin toxin has been detected in tissue sections, some tissue specimens, nasal swabs, and fluids by using immunologically based methods to study animals and animal tissues (DOD, 1999; Brzezinski and Craft, 2007). Immunologically based methods applied to human and animal fluid specimens have the potential to measure ricin concentrations as low as 0.1 ng/mL (1.54 pmol/L) (Shyu et al., 2002a,b). However, such applications have not been clinically validated, and concentrations after toxic exposures are unknown. Reference laboratories such as the US Army Medical Research Institute of Infectious Diseases (USAMRIID) (Aberdeen Proving Ground, MD) and the CDC are currently adapting these and other analytic methods for application to human specimens. At the US Army Combat Capabilities Development Command Chemical Biological Center, Aberdeen Proving Ground, Maryland (CCDC CBC, Aberdeen Proving Ground, MD), matrix-assisted laser desorption-ionization mass spectrometry is used as a definitive method for identification in biological specimens, but this technique is not currently able to provide quantitative results. Technologies used for assessing enzymatic activity are the cell-free luciferase-based assay, the cell-based apoptosis assay, and a recently developed liquid chromatography tandem mass spectrometry (LC-MS) technique (Zamboni et al., 1989; Roen et al., 2013). Another technique that has been described measures the liberation of radio-labeled adenosine from RIP substrates resulting from their enzymatic activity. This technique has similar sensitivity to the LC-MS method with a range of 0.01−10 pmol, but it involves the hazards of radio-labeled nucleotides and is not readily transferable to field-forward use (Brigotti et al., 1998).

28.9.2 DNA-based assays: polymerase chain reaction

PCR is a technique for logarithmically amplifying a DNA sequence to the extent that a sufficient quantity of material may be available to detect through either fluorescence or gel electrophoresis. The technique was developed in 1993 by Kary Mullis, who was awarded the Noble Prize in Chemistry that year for his pioneering work (Bartlett and Stirling, 2003). As a result of its simplicity, PCR is a highly popular technique with a wide range of applications, including direct nucleic acid sequencing, genomic cloning, DNA typing, the detection of infectious organisms, site-directed mutagenesis, parental genetic disease research, and the analysis of allelic sequence variations (Sambrook and Russell, 2001).

Typically, when PCR is used as an identification technique, the target sequence to be amplified is a sequence of DNA known to be specific to one particular organism; this is referred to as template DNA. The complementary strands of the double-stranded template are separated at 95°C, and the temperature is lowered slightly to allow two 10- to 30-base pair (bp) ssDNA primers to anneal to their complementary strands of the template. DNA polymerase binds the primer–template hybrid and initiates synthesis of DNA complementary to the template from the primer in a 5′–3′ direction. This cycle of heating to denature the double-stranded template DNA, followed by annealing and extension of the primers by DNA polymerase, is typically repeated approximately 30 times to yield literally billions of copies of the original template (Antwerpen et al., 2008).

In the original PCR process, described by Mullis, *E. coli* DNA polymerase was used for DNA synthesis. The challenge presented by using the enzyme was that the 95°C melting step destroyed its polymerase activity, and the enzyme had to be replenished after the heating stage cycle. As a result, the original Mullis PCR process was very laborious and required vast amounts of DNA polymerase. The problem was resolved in 1986 by utilizing DNA polymerase from *Thermophilus aquaticus* bacterium that was discovered in Colorado hot springs. The bacterium was well-adapted to the extremely high water temperatures of the springs, and its DNA polymerase was found to endure the high temperatures required for PCR (Munster et al., 1986). The enzyme allows a series of 30–40 cycles of PCR amplification without the need of replenishing the polymerase and also reduces the associated potential for introducing contamination into the PCR assay. Moreover, because of its thermophilic origins, Taq polymerase functions optimally at approximately 72°C, allowing DNA synthesis to be performed at much higher temperatures than was possible with the *E. coli* enzyme. Because of the higher stringency of PCR primer annealing at the higher temperatures, the DNA template is copied with much higher fidelity, and the nonspecific products that had affected many previous PCR reactions are reduced (Saiki et al., 1988). One of the major disadvantages of Taq polymerase, however, is its low replication fidelity. Taq polymerase does not have a 3′–5′ exonuclease proofreading mechanism to replace base-pairing mismatches in the newly synthesized DNA strand; however, it does have a 5′–3′ exonuclease activity that allows real-time PCR (Luthra et al., 1998). The intensity of fluorescence emitted during the PCR reaction is monitored in real-time PCR, acting as an indicator of the degree of PCR amplification occurring during each PCR cycle. Thus, with newer real-time PCR instruments, reaction progress can be observed visually in real time.

The CDC and Department of Homeland Security Laboratory Response Network centers conduct PCR tests and time-resolved immunofluorescence assays to detect the ricin toxin in environmental specimens. Immunofluorescence is the immunoassay technique that uses a detector antibody or an antigen labeled with florescent dyes (Lim et al., 2005). Time-resolved fluorescence simply delays the time between excitation of the fluorescent dyes and quantifying resulting emission. The long-lived emissions of the fluorescent dyes are exploited to reject background signals such as sample autofluorescence as well as Rayleigh and Raman scatter (Marriott et al., 1991). Detection of the delayed emission leads to an increase in the signal-to-noise ratio of the dye, thereby lowering the detection limit of the assay. Time-resolved fluorescence also enhances separation of emission spectra from multiple dyes, thus improving assays that are multiplexed (Yan and Marriott, 2003).

Cell-based bioassays are sometimes used to confirm the intact toxin-killing activity of ricin in environmental samples because PCR-based assays cannot directly detect the toxin protein (Brzezinski and Craft, 2007).

Many of these technologies have the potential to quickly and conveniently determine whether an unknown sample possesses ricin activity and could greatly aid in the triage of victims after suspected ricin exposure. Furthermore, it would enable determination of the effective decontamination for a site. Technology of this nature may also greatly aid in an initial forensic investigation by immediately determining potency, and field-forward personnel could estimate the purity, grade, and the age of the weapon. Finally, a rapid screening assay for RIP activity would also prove beneficial for the pharmaceutical industry as a screening tool for genetically engineered ricin developed for cancer chemotherapeutics, and for basic researchers attempting to identify and characterize new and novel RIPs.

28.10 Concluding remarks and future directions

The assays for ricin described in this chapter are based on either ELISA detection of the toxin or DNA-based detection. Largely ignored is the fact that the lectin portion (B-chain) of the intact toxin could also serve as an analytical target. Carbohydrate-binding lectins bind to carbohydrate ligands with weak binding affinities, ranging in molar to submillimolar concentrations, even when preserving high specificities of the interaction. Lectins have the ability to distinguish a wide range of carbohydrate structures on the surface of cells. Their importance in cell recognition as related to mitogenesis, agglutination, and direction of cell cytotoxicity has been established (Sharon and Lis, 1989).

This specificity has been exploited to differentiate various cancer cells (Welty et al., 2006). Our understanding of the adsorption and distribution of the ricin toxin within the target organism could be greatly enhanced through the use of such techniques.

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Chapter 29

Botulinum toxin

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29.1 Introduction

The specific focus of this chapter is on the use of botulism and/or botulinum toxins as weapons. Botulism is a disease caused by the neurotoxins produced by an anaerobic, spore-forming *Clostridium botulinum* bacteria found in soil. The most common forms of naturally occurring human botulism include foodborne, infant, and wound botulism. Foodborne botulism is the most common form of the naturally occurring disease in humans. Foodborne botulism is caused by consuming foods contaminated with botulinum spores, which germinate and multiply into bacteria to produce neurotoxins in the food. Important sources of foodborne botulism include improperly preserved home-processed foods such as honey, corn, green beans, and beets. Less frequent sources are fish products and commercially processed foods. Infant botulism is often associated with eating honey contaminated with spores, but new evidence suggests that soil and dust brought into the house from the outside may also be a significant source of botulinum spores. Wound botulism occurs when spores contaminate a wound, germinate, and produce neurotoxins that are then absorbed into the bloodstream.

Regardless of the form of botulism, disease results from the intoxicating effects of potent neurotoxins. Botulinum neurotoxins (BoNTs) comprise a family of seven distinct neurotoxic proteins produced by immunologically discrete strains of *C. botulinum*. This family of neurotoxins has an estimated human LD_{50} of 1−3 ng/kg (Simpson, 2004; Sobel et al., 2004). Due to their extremely high toxicity, ease of production, and previous history of weaponization, the BoNTs have been designated as category A threat agents by the US Centers for Disease Control and Prevention (CDC). Category A agents are defined by the CDC as those that "result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption and require special action for public health preparedness."

While there are currently seven known antigenic serotypes of BoNT, only serotypes A, B, and E are predominantly associated with human intoxication. Intoxication by BoNTs leads to bilateral flaccid paralysis, involving skeletal muscle and structures innervated by autonomic fibers (Simpson, 1986; Shapiro et al., 1998). Death is inevitable if the intoxication is left untreated. The toxicity of BoNTs leading to flaccid paralysis of skeletal muscle is due to their ability to block acetylcholine (ACh) release from peripheral cholinergic nerve endings. Paralysis could persist for weeks to months (depending on the serotype), and the available treatment consists of supportive care including fluids, total parenteral nutrition (TPN), and mechanical ventilation. Death occurs when the diaphragm and intercostal muscles become sufficiently compromised to impair ventilation or when patients succumb to secondary infections following long periods of intensive care (Hatheway et al., 1984; Shapiro et al., 1998). In survivors the effects of what effectively amounts to denervation associated muscular atrophy can be severe and long-lasting.

29.2 Historical aspects

The weaponization of BoNTs continues to pose a serious concern. The toxins are highly lethal, easy to isolate, and easy to deliver. The modern development of BoNTs as offensive weapons began in the 1930s. During the occupation of Manchuria the Japanese fed lethal cultures of *C. botulinum* to prisoners as part of experiments conducted by its biological and chemical weapons program (Unit 731) (Harris, 1994; Hill, 1947). In response to intelligence indicating that Germany attempted to weaponize botulinum

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toxins as a defense against invasion the United States formed the War Research Service in 1942. This service investigated the potential use of Anthrax and botulinum toxin-based weapons. At this time, the specific toxic agents and their mode of action were not fully understood (Dickson and Shevky, 1923). Thus the goals of the earliest research were to isolate and purify the toxin and determine its mode of action (Guyton and MacDonald, 1947). By June 1944 the United States had stockpiled sufficient quantities of botulinum toxin to allow for massive retaliation if German forces attempted to use biological agents in the European theater of World War II (Cochrane, 1947; Bernstein, 1987, 1990; Franz et al., 1997). The US code name given to BoNT at that time was "agent X." Subsequent work was conducted by the US at Camp Detrick. Based on President Richard M. Nixon's 1969–1970 executive orders (explicitly stated in National Security Decision Memoranda 35 and 44, 13) the US's offensive weapon stockpiles of BoNT were subsequently destroyed (Tucker and Mahan, 2009; Lebeda, 1997).

In 1975 the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction came into force (OPBW, 1975). This convention prohibited the production of offensive toxins, including botulinum toxins. While the Soviet Union signed and ratified the convention, its biological warfare program, which included BoNT research, biological weapons development, and production, continued and was expanded in the post-Soviet era (Adams, 1995; Bozheyeva et al., 1999). Testing of botulinum-filled weapons occurred at the Soviet Aralsk-716 test site on Vozrozhdeniye (Renaissance) Island in the Aral Sea (Bozheyeva et al., 1999; Miller, 1999). The Soviet Union also attempted to transfer complete botulinum toxin genes into other bacteria (Alibek and Handleman, 1999). During the presidency of Boris Yeltsin, Russia, despite its treaty obligations, continued a large covert effort to effectively weaponize botulinum toxins (Alibek and Handleman, 1999).

Despite also being a signatory to the Biological Weapons Convention, Iraq acknowledged that it produced 19,000 L of concentrated BoNT and incorporated this material into weapons systems (UN, 1991; Zilinskas, 1997; Blix, 2004). Weapon delivery systems included 13 SCUD missiles, bombs, and tank sprayers. However, fortunately, these weapon systems were never used in combat. Iraq has subsequently claimed that its biological warfare stockpiles were destroyed.

Nonstate-sponsored terrorists have an interest in BoNTs (Brackett, 1996). Members of the Japanese Aum Shinrikyo cult made attempts to develop BoNT-based weapons following the political defeat of the party during the 1990 Japanese elections. While the cult is best known for the subway sarin attack in Tokyo in 1995, the organization was also responsible for an attempted biological weapons attack on the Kasumigaseki subway station 24 in the same year (Danzig et al., 2011; Sugishima, 2003). Investigations into the cult demonstrated that the organization was unable to effectively produce and weaponize BoNTs because the organization lacked the scientific expertise to correctly culture *C. botulinum* and to preserve the toxins.

Investigators have attempted to evaluate the consequences of a BoNT attack by a terrorist group by evaluating what would have happened if the Rajneeshee cult (the followers of the Bhagwan Shree Rajneesh who had carried out a biological attack to influence a local election) had used botulinum toxin instead of *Salmonella typhimurium* on salad bars in its 1984 attack in The Dalles, Oregon (Miller et al., 2001; Smith, 2006). Their conclusions were that many of the 751 who were poisoned during the incident would likely have died and the event would have very quickly overwhelmed the locally available medical resources.

Other approaches have been to model the effects of the 2006 foodborne botulism outbreak in Thailand (Ungchusak et al., 2007; Kongsaengdao et al., 2006; Witoonpanich et al., 2010; Hardin and Cohen, 1988; Dembek et al., 2007). During this outbreak 209 individuals were exposed to BoNT-contaminated food, of which 163 required medical examination, 140 required hospitalization, and 42 required respiratory support. Thailand lacked sufficient local stocks of BoNT antitoxin and stocks of these materials had to be donated and imported. The available donated supplies were only sufficient to treat 90 patients. For logistical reasons antitoxin treatment was delayed for 5–9 days following poisoning. The median duration of hospital admission was 6 days for patients without mechanical ventilation and 25 days for patients with mechanical ventilation. This incident demonstrated that apart from the technical and infrastructure needs associated with the provision of respiratory support, the management of mass BoNT incidents requires a complex range of medical expertise including neurologists, pulmonologists, respiratory care technicians, intensivists, cardiologists, infectious disease specialists, pharmacists, mental health specialists, and rehabilitation and referral services.

Wein and Liu (2005) mathematically modeled a BoNT attack utilizing the US milk supply. Because of biological and logistical factors milk represents a potentially ideal medium to disperse BoNTs amongst a large population. However, a significant limitation of this exposure scenario is that it may not adequately account for the use of pasteurization within the US milk supply chain, that is, introduction of BoNTs into the supply chain before pasteurization would likely substantially reduce the impact of such an attack (standard pasteurization at 72°C

for 15 s inactivates at least 99.99% of BoNT/A and BoNT/B and at least 99.5% of their respective complexes). Given how the US milk supply chain operates, incorporating large amounts of BoNTs into the supply following pasteurization with the objective of a widely dispersive bioterrorism attack is regarded as being difficult. Other exposure scenarios that have been modeled include BoNT distribution using a public drinking fountain and within a recreational facility (Dembek et al., 2005).

29.3 Background

29.3.1 Toxin structure and molecular function

The highly potent neurotoxins synthesized by the *C. botulinum* microorganism and several related clostridial species (*C. baratii*, *C. butyricum*, and *C. argentinense*) are the causative agents of botulism, a potentially lethal disease historically associated with the ingestion of contaminated food products. Seven different BoNTs, designated A through G, are currently known to be produced by various strains of clostridial bacteria; these neurotoxins are antigenically distinct but comparable in basic structure. The BoNTs are members of a superfamily of homologous proteins that also include tetanus neurotoxin. BoNTs are generated as single-chain polypeptides that are then posttranslationally modified (i.e., proteolytically nicked) to yield a disulfide bond-linked dichain structure composed of a heavy chain (H-chain or HC) and a light chain (L-chain or LC). Enzymes synthesized by these microorganisms themselves often mediate this cleavage, although the gastrointestinal enzymes of the host can also generate the dichain structure from the ingested toxin. The three-dimensional dichain protein structure of the purified toxin is provided (Fig. 29.1).

29.3.1.1 Function of heavy and light chains

The HC and LC of BoNTs each play critical roles in toxicity. HC is thought to mediate binding and internalization of the toxin at peripheral nerve synapses. LC, the toxic moiety, inhibits neurotransmitter exocytosis through its zinc-dependent endoproteolytic activity. The LCs of the various BoNT serotypes differ in their distinct molecular targets within the peripheral cholinergic nerve terminals (Schiavo et al., 1992, 1993a,b; Blasi et al., 1993). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects, despite their distinct targets.

BoNT serotype A is the most well characterized of the different serotypes in terms of both structure and function. Early biochemical efforts led to its crystallization, and this crystalline form was used in numerous animal studies

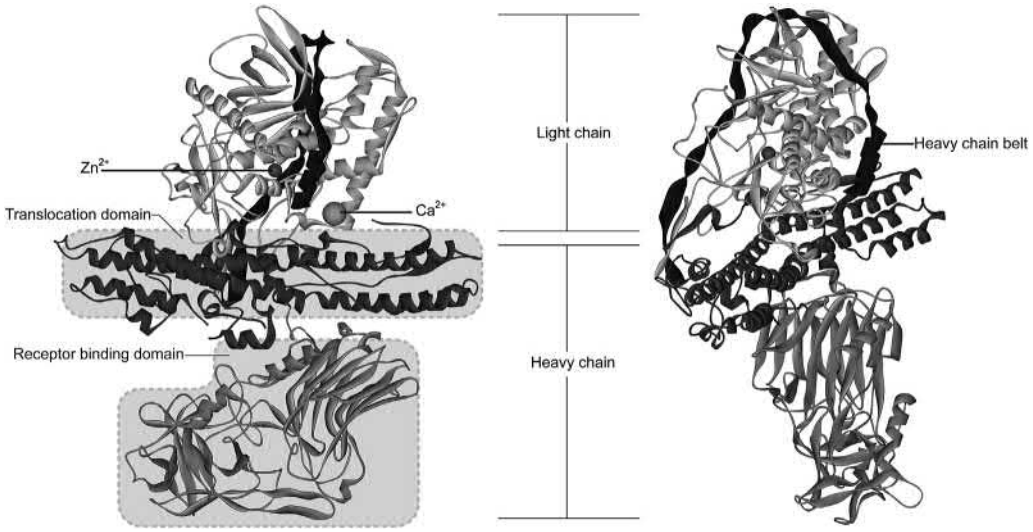

FIGURE 29.1 Three-dimensional structure of botulinum toxin serotype A (BoNT/A). BoNT/A (1296 amino acids), rendered as a ribbon structure, is depicted in two views. BoNTs are synthesized as a single polypeptide and nicked by bacterial proteases to form a dichain molecule. The 50-kDa LC, with 448 residues, and the 100-kDa HC, with 848 residues, are linked by a disulfide bond. All BoNTs comprise three major domains: a receptor-binding domain (C-terminal end of HC), a translocation domain (N-terminal end of HC), and a zinc-binding metalloprotease domain on LC. All seven BoNTs exhibit conserved sequences, but they are also antigenically distinct at the same time. The LC seems to be held in place by the translocation belt of HC (Brunger et al., 2007). The belt spans residues 492–545 in BoNT/A and 481–532 for BoNT/B and wraps around the catalytic domain of the LC. Brunger et al. (2007) suggest that the belt acts as a surrogate pseudosubstrate inhibitor of the LC protease and acts as a chaperone during translocation across the endosome membrane into the cytosol. The belt occludes access to the active site of LC, thereby holding the unreduced holotoxin in its catalytically inactive state. The sphere represents the bound Zn^{2+} at the LC active site. The structure of BoNT/A holotoxin was provided free of copyright restrictions from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Berman et al., 2000; PDB ID: 2nz9; Garcia-Rodriguez et al., 2007) and rendered using Accelrys DS Visualizer 2.0 software.

on the pathogenesis of botulism. Crystalline type A toxin has a sedimentation constant of 19S (around 900 kDa), which is far larger than the combined size of the HC and LC components (Simpson, 1981). This large progenitor toxin form was shown to dissociate under moderately alkaline conditions, releasing the derivative (7S) or neurotoxin component (Heckly et al., 1960; Sugii et al., 1977a, b; Chen et al., 1998). The derivative neurotoxin has a molecular weight of 150−160 kDa, representing the combined size of the H- and L-chains. Additional work led to the finding that the dichain molecules comprising the various neurotoxins are often released as higher-order polypeptide complexes. The crystalline type A progenitor toxin consists of the 7S neurotoxin and one or more noncovalently linked accessory proteins. These nontoxic components of the progenitor toxin complex were later identified as three different hemagglutinins (HAs) and a nontoxic nonhemagglutinin (NTNH) protein.

29.3.1.2 Accessory proteins of the progenitor toxin complex

The accessory proteins of the progenitor toxin serve to enhance the stability of the toxin to ensure uptake from the gut. The NTNH component of the multimeric type A toxin complex is encoded by a single gene upstream of the neurotoxin locus, while three different HA proteins have been characterized in association with BoNTs. All *C. botulinum* serotypes have been shown to produce neurotoxin complexes with the NTNH and HA proteins.

29.3.2 Overview of botulinum neurotoxin action

After gaining entry to the lymphatics and circulation via the gastrointestinal tract, BoNTs function as potent neuromuscular blockers. The cellular and molecular mechanisms involved in toxin absorption, transit to specific target tissues, escape from the vasculature, and uptake within peripheral cholinergic nerve terminals have yet to be fully characterized. However, each of the neurotoxins has been shown to block vesicular neurotransmitter release at peripheral cholinergic synapses through the endoproteolytic cleavage of proteins associated with the exocytosis machinery (Schiavo et al., 1993a,b, 1995; Blasi et al., 1993). At peripheral cholinergic nerve endings, BoNT binding to high-affinity receptors leads to acceptor-mediated endocytosis and low pH-induced translocation across the endosomal membrane into the cytosol. The carboxy-terminal region of the toxin HC appears to mediate binding at the nerve synapse, while the amino-terminal domain controls translocation. The LC is held in close association with the HC by an amino acid belt. The LC of each toxin functions as a zinc-dependent endoprotease, cleaving at least one of three soluble *N*-ethylmaleimide-sensitive fusion protein attachment receptor (SNARE) proteins involved in neurotransmitter release.

Stimulus-evoked, calcium-dependent release of ACh from the cholinergic synapse normally occurs through the formation of a fusion complex between ACh-containing vesicles and the intracellular leaflet of the nerve terminal membrane (Arnon et al., 2001). This synaptic vesicle (SV) fusion complex consists of several proteins of the SNARE family, including a 25-kDa synaptosomal associated protein (SNAP-25), vesicle-associated membrane protein (VAMP, or synaptobrevin), and the synaptic membrane protein syntaxin. Other SNARE proteins have been identified as components of membrane transport systems in yeast and mammals but have not been implicated as targets for BoNTs. Meanwhile, type A and E neurotoxins cleave SNAP-25, while types B, D, F, and G act on VAMP and type C1 toxin cleaves both syntaxin and SNAP-25. Neurotoxin-mediated cleavage of any of these substrates disrupts the processes involved in the exocytotic release of ACh and leads to flaccid paralysis of the affected skeletal muscles.

29.3.3 Clinical forms of botulism in humans and animals

Exposure to BoNTs can produce lethal disease in humans and other animal species. Six different clinical forms of human botulism have been described in the literature (see Table 29.1), including (1) foodborne botulism, (2) infant botulism, (3) wound botulism, (4) an adult form of infant botulism, (5) inadvertent systemic botulism, and (6) inhalation botulism (reviewed by Cherington, 1998; Middlebrook and Franz, 2000; Arnon et al., 2001). Botulism is the result of either an infectious process involving elaboration of toxin from the colonizing clostridial organism, or a noninfectious process. Infant and wound botulism are the most prevalent infectious forms of botulism. Although rare, an adult form of infant botulism has been documented; gastrointestinal colonization in adults may be enabled by alterations in normal gastrointestinal flora resulting from antibiotic treatment (Cherington, 1998). Foodborne and inhalational exposures have noninfectious etiologies and are the result of ingesting or inhaling preformed toxin. Although only one inhalational botulism incident has been reported in humans, this incident demonstrates that humans are susceptible to respiratory intoxication similar to that which has been experimentally produced in many laboratory species (reviewed by Middlebrook and Franz, 2000). Finally, the emergence of a multitude of therapeutic applications for BoNTs has led to infrequent cases of inadvertent systemic botulism resulting from local toxin injection (reviewed by Arnon et al., 2001).

TABLE 29.1 Clinical forms of botulism.

	Infectious			Noninfectious		
	Infant	**Wound**	**Adult colonization**	**Foodborne**	**Inhalational**	**Inadvertent systemic**
Cause	Colonization of immature intestinal tract	Wound colonization resulting from contact with contaminated material	Intestinal colonization secondary to disruption of normal intestinal flora	Ingestion of preformed toxin in contaminated food products	Respiratory exposure to toxin aerosols or droplets	Systemic toxin uptake after therapeutic toxin administration
Susceptibility	Young infants (2–4 months of age) prior to establishment of normal intestinal flora	Self-administering users of i.v. drugs (often black tar heroin)	Antibiotic-treated patients	All exposed individuals	All exposed individuals	Patients treated with local toxin injections

29.3.4 Infectious forms of botulism

29.3.4.1 Infant botulism

Infectious botulism is a consequence of ingesting or inhaling clostridial spores that colonize the large intestines, germinate, and elaborate toxin into the bloodstream. Infant and wound botulism are the most prevalent infectious forms of botulism. Infant botulism comprises the majority (72%) of reported human botulism cases in the United States, while most of the remaining cases involve foodborne and wound botulism (Mackle et al., 2001). Infants are especially susceptible to infant botulism. Infants were found to be uniquely susceptible to gastrointestinal colonization due to a lack of well-established competing gut flora (Arnon et al., 1995). While infant botulism can be acquired by inhaling spores, this differs markedly from inhalational botulism, which results from inhaling preformed aerosolized toxin and not spores. Clostridial spores do not pose a threat in older infants or most adults (Arnon et al., 1995, 1998; Cox and Hinkle, 2002).

29.3.4.2 Wound botulism

Wound botulism involves growth of *C. botulinum* spores in a contaminated wound with in vivo toxin production (Weber et al., 1993). It accounts for less than 25% of all botulism cases. The majority of wound botulism cases are caused by serotype A and the remainder by serotype B (Shapiro et al., 1998). The neurological symptoms of wound botulism differ little from those of foodborne botulism except for the general absence of gastrointestinal symptoms. From its discovery in 1943 until 1996, only 111 incidents of wound botulism were documented (Merson and Dowell, 1973; CDC, 1998; Shapiro et al., 1998); among the 100 laboratory-confirmed cases, 83 cases were type A, 16 cases type B, and one a mixture of type A- and B-producing organisms (CDC, 1998). Risk factors for wound botulism include deep wounds, avascular areas, compound fractures, and crush injuries of the hand. Although a rare form of naturally occurring BoNT intoxication, it most recently occurred in Maryland as a result of a construction worker receiving a contaminated, compound fracture of the femur after falling into an excavated pit (Hilmas, personal observation). Wound botulism also occurs in intravenous (i.v.) drug users as a result of bacterial colonization at needle puncture sites or nasal/sinus lesions secondary to cocaine snorting (MacDonald et al., 1985). From 1986 through 1996, 78 cases of wound botulism were reported, and the majority of cases were linked to black tar heroin, introduced intravenously.

29.3.4.3 Child or adult botulism from intestinal colonization

Gastrointestinal colonization in adults or children by clostridia bacteria does not typically take place except in circumstances where the normal flora has been altered by antibiotic treatment (Cherington, 1998). Botulism results from in vivo production of toxin, analogous to the pathogenesis of infant botulism (Chia et al., 1986). Support for this form of botulism is provided by the demonstration of prolonged excretion of toxin and *C. botulinum* in stool, by the demonstration of *C. botulinum* spores but not preformed toxin in suspected foods, or both.

29.3.5 Noninfectious forms of botulism

29.3.5.1 Foodborne botulism

Worldwide, BoNT intoxication is most commonly associated with food poisoning. In the early 19th century, the effects of botulism were observed to be associated with the consumption and handling of meat products. Thus, German physician Justinus Kerner described what he termed "sausage poisoning" (Erbguth, 2004). It was later in the 19th century that the term *botulism* was used, from the Latin *botulus*, meaning "sausage." Foodborne botulism results from ingesting preformed toxin in food contaminated with toxin spores. Inadvertent and inhalational botulism, two other noninfectious forms of botulism, also involve exposure to preformed toxin. Outbreaks of foodborne botulism in the United States result from eating improperly preserved home-canned foods (CDC, 1995). The majority of cases of foodborne botulism are due to serotypes A, B, and E. From 1990 through 1996, type A accounted for 44.6% of foodborne outbreaks in the United States, followed by type E (35.7%) and type B (12.5%) (CDC, 1998). The prompt recognition of such outbreaks in the United States and early treatment with serotype-specific botulinum antitoxin has limited the number of casualties, severity of the disease, and the case-to-fatality ratio. Mortality from foodborne botulism has declined from 60% (CDC, 1998) in 1950 to less than 10% of clinical cases (Shapiro et al., 1998).

29.3.5.2 Inhalational

Because humans are relatively resistant to gut colonization by the *C. botulinum* microorganism, oral and inhalational exposures to preformed neurotoxin are likely to present the greatest threats with respect to intentional dissemination. The ability for inhaled BoNTs to produce illness has been documented in humans and in several experimental species. Only one incident involving inhalational intoxication in humans has been reported. Three laboratory workers presented with physical and neurological symptoms after accidental respiratory exposure to aerosolized type A toxin (Middlebrook and Franz, 2000; Arnon et al., 2001). These patients were all successfully treated with antiserum, gradually recovering from their weakness and visual disturbances over the next several days. After inhalational exposure, the neurotoxins are absorbed from the respiratory tract into the lymphatics and circulation for transport to peripheral cholinergic synapses (reviewed by Simpson, 2004). The pathogenesis following neurotoxin absorption is thought to be similar for both the respiratory and gastrointestinal exposure routes. Thus, the primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism.

29.3.5.3 Inadvertent systemic botulism

The therapeutic indications for BoNTs are numerous. They are used in the treatment of ophthalmological disorders (strabismus, Duane's syndrome, esotropia/exotropia), movement disorders (focal dystonias, blepharospasm), spasticity, neuromuscular disorders, pain (headache, myofacial pain), disorders of the pelvic floor (anal fissures), ear/nose/throat disorders, cosmetic applications (wrinkles), and hyperhidrosis. The recent explosion in new indications for BoNTs in the treatment of a wide range of medical conditions also brings the possibility of medical errors in BoNT dosing. Systemic botulism may result from injection of excessive doses of the potent neurotoxin. The most infamous case of systemic botulism involved the paralysis of four Florida patients, including a doctor, who were treated with BoNTs for wrinkles. The physician used formulations of type A from Toxin Research International, Inc., which were approved by the US Food and Drug Administration (FDA). The research grade type A neurotoxin was apparently sold to the doctor and reconstituted to be thousands of times more potent than the typical dose used in BOTOX for paralyzing facial muscles. Later testing estimated that the raw bulk toxin that was used contained between 20,000 and 10 million units of botulinum toxin. In comparison, a typical vial of BOTOX from Allergan, Inc. contains only 100 units (CIDRAP (Center for Infectious Disease Research and Policy, 2004)). All three patients and the physician were injected with the toxin preparation; they developed severe systemic botulism requiring mechanical ventilation. While all four survived the superlethal dose of type A toxin, several of the patients experienced a syndrome involving chronic gastrointestinal symptoms and discomfort months after exposure.

29.3.6 Human intoxication

The basic syndrome of BoNT intoxication is similar for all naturally occurring forms, as well as for inhalation exposure, and does not vary appreciably among serotypes (Hatheway et al., 1984; Simpson, 1986; Jankovic and Brin, 1997). Based upon documented laboratory evidence, human BoNT intoxication is caused by exposure primarily to serotypes A, B, E, and (to a much lesser extent) to serotype F; disease manifests mostly as a result of foodborne, infant, and wound botulism (Simpson, 1986). BoNTs are also lethal when inhaled in aerosolized form, although this is not generally observed in nature.

The various toxin serotypes are usually associated with analogous clinical presentations. Paralysis proceeds in a descending fashion after an initial bulbar involvement. The earliest symptoms of botulism typically include visual disturbances, followed by dysphagia, dysphonia,

and dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action (Jankovic and Brin, 1997). A descending generalized skeletal muscle weakness may then develop, progressing from the upper to the lower extremities. Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death unless appropriate supportive care is provided (Shapiro et al., 1998).

29.4 Epidemiology

29.4.1 Foodborne botulism

Human foodborne botulism outbreaks have typically been linked to the consumption of toxin-contaminated home-prepared or home-preserved foods (Maselli, 1998). The vast majority of foodborne botulism cases are attributed to toxin types A, B, or E. Maselli (1998) reports that type B is the most prevalent (52%) in the United States, followed by type A (34%) and type E (12%), while the CDC (1998) suggests 37.6% of all foodborne botulism outbreaks since 1950 were caused by type A, 13.7% by type B, 15.1% by type E, 0.7% by type F, and 32.9% were unidentified with respect to toxin type. Outbreaks of type F and G botulism are rare (Sonnabend et al., 1981; Maselli, 1998; Richardson et al., 2004), and only anecdotal reports of isolated type C1 and D botulism cases can be found in the published literature (e.g., Lamanna, 1959).

The natural epidemiology of foodborne botulism provides additional insight into the similarities and discrepancies between the human disease and that represented in various animal models. In the United States, around 25% of reported human botulism cases are classified as foodborne and 72% are infant (Mackle et al., 2001). Human type A and B foodborne botulism cases occur worldwide and constitute the vast majority of reported human intoxications (Maselli, 1998). The majority of other botulism cases are attributed to serotype E and are typically associated with the consumption of contaminated seafood. Generalizations have been made regarding the geographic distribution of the most common *C. botulinum* strains within the United States. Most human foodborne botulism outbreaks occurring west of the Mississippi are due to type A toxin; type B strains are more prevalent east of the Mississippi, while type E strains are typically isolated to Alaska and the Pacific Northwest (Arnon et al., 2001; Richardson et al., 2004).

Several clinical and epidemiological reports have evaluated the worldwide geographic distributions of human foodborne botulism cases. A review of 13 outbreaks between 1970 and 1984 identified geographic differences in the toxin serotypes associated with human foodborne botulism cases. Type B botulism was predominant in Portugal, Spain, France, and several other European countries (Lecour et al., 1988). Interestingly, the low mortality rate associated with human type B foodborne botulism (8.8%, vs 24.3% for type A and 30.8% for type E in the United States from 1950 to 1979) did not correlate with the high oral toxicity for type B toxin in mice (Ohishi et al., 1977; Sugii et al., 1977a,b,c). Serotype E was linked to botulism outbreaks in select regions such as the Baltic countries (Lecour et al., 1988) and typically resulted from the consumption of contaminated fish (Maselli, 1998).

Type F toxin was associated with only two reported outbreaks of human foodborne botulism prior to 1998 (Maselli, 1998). The first of these outbreaks occurred in Denmark (on the island of Langeland) and was attributed to a contaminated liver paste product (Richardson et al., 2004). The second outbreak, in 1966, affected three individuals in California and was associated with homemade venison jerky (Richardson et al., 2004). While a few other type F botulism cases have been reported, they are generally thought to have resulted from intestinal colonization and type F toxin production by another related species, *C. baratii* (Richardson et al., 2004). A recent report of a type F botulism case in California provided some additional insight into this uncommon toxin serotype and the associated clinical disease (Richardson et al., 2004). The patient described in this report presented with typical signs and symptoms, including ptosis, dysphagia, and weak extremities. Although the source of the ingested toxin was not conclusively determined, the exposure was tentatively linked to shellfish consumption, and type F toxin was subsequently detected in the patient's stool (Richardson et al., 2004). Human type F botulism cases may have been underreported in the past since some laboratories did not test culture isolates for the presence of *C. baratii*, which also produces type F toxin.

Type G toxin-producing clostridial organisms (*C. argentinense*) have been detected in several soil samples from a South American cornfield (Maselli, 1998), but only one reported outbreak of type G botulism (in Switzerland) has been identified in the published literature (Sonnabend et al., 1981). Certain aspects of this outbreak draw questions as to whether it was truly associated with type G intoxication. Type G organisms were isolated from all four affected adults and an 18-week-old infant, suggesting that the intoxications were due to ingestion and subsequent colonization by type G spores (Sonnabend et al., 1981). Type G toxin was detected at low levels of two to seven mouse intraperitoneal lethal dose 50 ($MIPLD_{50}$)/mL in the serum of three out of the four lethally intoxicated adults, all of whom died suddenly sometime after the presumed foodborne intoxication. Type A toxin was also detected in two of these individuals, suggesting that the intoxications may have involved colonization either by a mixed set of clostridia or by a unique strain producing multiple toxins

(Sonnabend et al., 1981). Alternatively, detection of dual serotypes could have been an artifact of the culture or testing methods. Soil samples taken from the area indicated the presence of only type A clostridial organisms (Sonnabend et al., 1981). Regardless, the occurrence of human type G botulism is rare, and the relative susceptibility of humans to colonization and intoxication from this serotype is not clear.

Species-specific patterns of susceptibility to different toxin types are common in both naturally occurring and experimental foodborne botulism. These differences do not necessarily facilitate identification of the most appropriate animal models from the human condition, but they may help to eliminate highly variant species. For example, mink were reported to be relatively resistant to toxin types A, B, and E (Yndestad and Loftsgard, 1970), which are responsible for the vast majority of human botulism outbreaks (Maselli, 1998; Arnon et al., 2001). Weanling pigs, on the other hand, were shown to be moderately resistant to types A, C1, E, and F and highly resistant to type D toxin (Smith et al., 1971). Experimental and epidemiological studies have identified one persistent difference in the epidemiology of botulism in humans compared to many other animal species. Few reports citing human outbreaks of types C and D are available. One of these reports mentions two type C outbreaks and one type D outbreak in humans, but provides no source for these cases (Lamanna, 1959). More recent reports of human types C or D botulism have not been found in the literature, and it is widely assumed that human foodborne intoxications are rarely, if ever, associated with these toxin types. In contrast, naturally occurring botulism of both types is quite common among domestic and wild animal species, and several studies have established the susceptibility of various laboratory species to experimental types C and D botulism.

The effect of C and D toxin serotypes, as well as A, B, and E, on human intercostal muscle have been studied (Anderson and Hilmas, 2015). All serotypes showed a similar ability to produce complete muscular paralysis in ex vivo human intercostal muscle. Intercostal muscle was excised from patients receiving a thoracotomy and intercostal muscle flap procedure. The muscle was removed tendon to tendon by surgical excision without electrocautery and dissected into multiple bundles with their associated intercostal nerves. The nerve−muscle units were placed in a vertical twitch bath and stimulated at 0.03 Hz (0.2-ms pulses of supramaximal strength) using a novel nerve clamp electrode to illicit an indirect muscle twitch. Potent toxins (1 nM) from various serotypes were added to the bath after confirming the stability of control muscle responses. In each case, twitch tensions declined to negligible amplitudes by 1 h after direct toxin application to the tissue bath.

Several nonhuman primate species are known to be susceptible to types C1 and D toxins both in nature and as experimental models. A large natural outbreak of type C botulism was reported in a troop of captive *hamadryas* baboons in 1989 (Lewis et al., 1990). The outbreak resulted in the deaths of 36 animals, including 3 adult males, 6 subadult males, 17 adult females, and 10 subadult females. Additional animals displayed mild to moderate symptoms that resolved themselves over a period of several days (Lewis et al., 1990). As with human foodborne botulism, various age groups and both sexes were affected, and no macroscopic lesions were apparent. Serum samples and gastric contents taken from ill animals contained type C1 toxin, although the source of the toxin was not identified (Lewis et al., 1990). The authors speculated that humans are probably also susceptible to type C1 toxin. The reason for the relative lack of human type C botulism cases remains unknown. It has been suggested that serotype C is often associated with carrion, providing a possible explanation for the absence of reported human cases. At least two other outbreaks of naturally occurring type C botulism in nonhuman primates were previously reported, one in squirrel monkeys (*Saimiri sciureus*) and capuchin monkeys (*Cebus capucinus* and *Cebus olivaceus*) (Smart et al., 1980) and the other in gibbons (*Hylobates lar*) (Smith et al., 1985).

In addition to nonhuman primates, most other animal species that show some sensitivity to botulinum intoxication are in fact susceptible to toxin serotypes C1 and D. Several rodent species are susceptible to oral intoxication with most botulinum toxins, including types C1 and D (Jemski, 1961a,b; Cardella et al., 1963; Sergeyeva, 1966; Sugiyama et al., 1974; Smith, 1986; Gelzleichter et al., 1999; Middlebrook and Franz, 2000). The majority of botulism outbreaks in cattle have also been attributed to toxins C1 and D. Cattle intoxication is typically associated with the ingestion of contaminated bones and other carcass remains; their apparent susceptibility to types C and D botulism might simply be due to frequent ingestion of decaying material that is primarily contaminated with these toxin types. A recent study indicated that cows are also uniquely sensitive to i.v. injection of type C1 toxin (Moeller et al., 2003). The high susceptibility of cattle to type C botulism is not dependent on exposure route, although the specific factors contributing to their sensitivity are not known.

A recent outbreak of type C botulism among farmed mink and foxes in Finland underscores the need to consider not only the quantitative susceptibility of various species to the toxins but also the potential epidemiological significance of interspecies differences in dietary patterns. Lindstrom et al. (2004) reviewed the Finland incident, which was the largest documented type C botulism outbreak in fur production animals. Over 52,000

animals developed illness after consumption of feed product that was contaminated with over 600 MLD of type C1 toxin per gram. This feed consisted of acidified slaughter by-products from poultry, beef, and fish (Lindstrom et al., 2004). According to national regulations, these by-products were acidified with an organic acid to yield a final pH of 4.0 or lower. Such processing would inhibit the growth of many microorganisms but would not necessarily result in significant toxin inactivation. Over 44,000 of the 52,000 affected animals died, and the death rate among all potentially exposed animals was almost 22% (Lindstrom et al., 2004).

The large number of animals affected and the high lethality associated with the outbreak could be considered indicative of the high susceptibility of the affected species to foodborne type C botulism. This high susceptibility might appear to be in stark contrast to that of humans due to the scarcity of type C cases in humans. However, the Finland outbreak provides a clear indication that dietary differences between species may play a significant role in these epidemiological patterns. Humans would be far less likely to consume slaughter by-products (including intestinal tissues), as opposed to higher-quality beef, poultry, and fish products. Moreover, the preparation of such products for human consumption would generally involve cooking rather than acidification. Thus, the influence of dietary habits must be taken into consideration when evaluating interspecies differences in epidemiological patterns for the various toxin serotypes.

It remains possible that humans generally do not consume the types of foods that are typically subject to contamination with type C1 and D toxins. Some researchers continue to speculate that humans are likely to be susceptible to both serotypes because they lead to botulism in monkeys, both in nature and after experimental oral exposure. Alternatively, humans might display a unique pattern of physiological susceptibility to the different toxin types. Lack of human susceptibility to type C1 and D intoxication could be attributed either to poor absorption of these specific toxins from the human gastrointestinal tract or to resistance of human cholinergic nerve terminals to the activity of these toxins. One cell culture study provided some support for the latter explanation. Type C1 neurotoxin was shown to bind with high efficiency to mouse neuroblastoma cells and to hybridomas of mouse neuroblastomas and rat gliomas, but not to human neuroblastoma cell lines (Yokosawa et al., 1989). Yokosawa et al. (1989) suggested that reduced binding of type C1 toxin to human versus mouse neuroblastoma cells could provide one explanation for the lack of type C botulism cases in humans.

Another potential explanation for the unique epidemiology of human botulism was provided in a study of botulinum toxin binding and transcytosis across polarized monolayers of two human colon carcinoma cell lines (T-84 and Caco-2). Substantial binding of iodinated BoNT/A and BoNT/B to human colon carcinoma cells was observed, while minimal binding of type C1 neurotoxin was detected (Maksymowych and Simpson, 1998). Both types A and B neurotoxins were also efficiently taken up, transcytosed, and released, by the polarized human carcinoma cells, whereas minimal transcytosis of type C1 neurotoxin was observed. The patterns of neurotoxin transcytosis (A and B, but not C1) observed in these human gut epithelial cell lines correlate with human susceptibility to foodborne botulism (Maksymowych and Simpson, 1998). The authors speculated that since human tissues are fully sensitive to the neuromuscular blocking properties of C1 neurotoxin (Coffield et al., 1997; Eleopra et al., 2004), the relative absence of human foodborne type C botulism could be due to the inability of this toxin to penetrate from the gut to the general circulation. Human susceptibility to type C1 and D neurotoxins remains unclear; however, clarification of this issue will be important in interpreting data derived both from in vitro studies on toxin transcytosis and from animal models for oral intoxication.

29.5 Pathogenesis

29.5.1 Overview of pathogenesis

BoNTs are a group of immunologically distinct but closely related bacterial proteins that act as potent inhibitors of synaptic transmission in skeletal muscle. Inhibition of ACh release from the presynaptic terminal of the neuromuscular junction (NMJ) is thought to be the sole mechanism involved in the toxins' lethal action (Simpson, 1986) and therefore the cause of botulism. The pathogenesis of intoxication is not completely understood but is generally thought to involve a multistep process to interrupt the normal vesicular release of ACh from the presynaptic motor nerve terminal. In a process of transcytosis, ingested or inhaled BoNT must first cross a barrier (either intestinal or pulmonary epithelial cells) to gain access to the circulation (see intestinal absorption of BoNT; Fig. 29.2). Once in the circulation, BoNT travels to its major target, the presynaptic membranes of alpha motor neurons at NMJs and neuroeffector junctions. Toxin binding through its HC to a cell surface receptor on the presynaptic motor nerve ending is followed by internalization via an endocytotic vesicle, acidification of the endosome, conformational change allowing cleavage of the enzymatically active LC from a bound HC, and release of LC toxin into the cytoplasm. Here, the LC cleaves one of the integral members of the SNARE complex (SNAP-25, VAMP, or syntaxin), proteins involved in exocytosis of ACh (Simpson, 1986, 2004; Black and

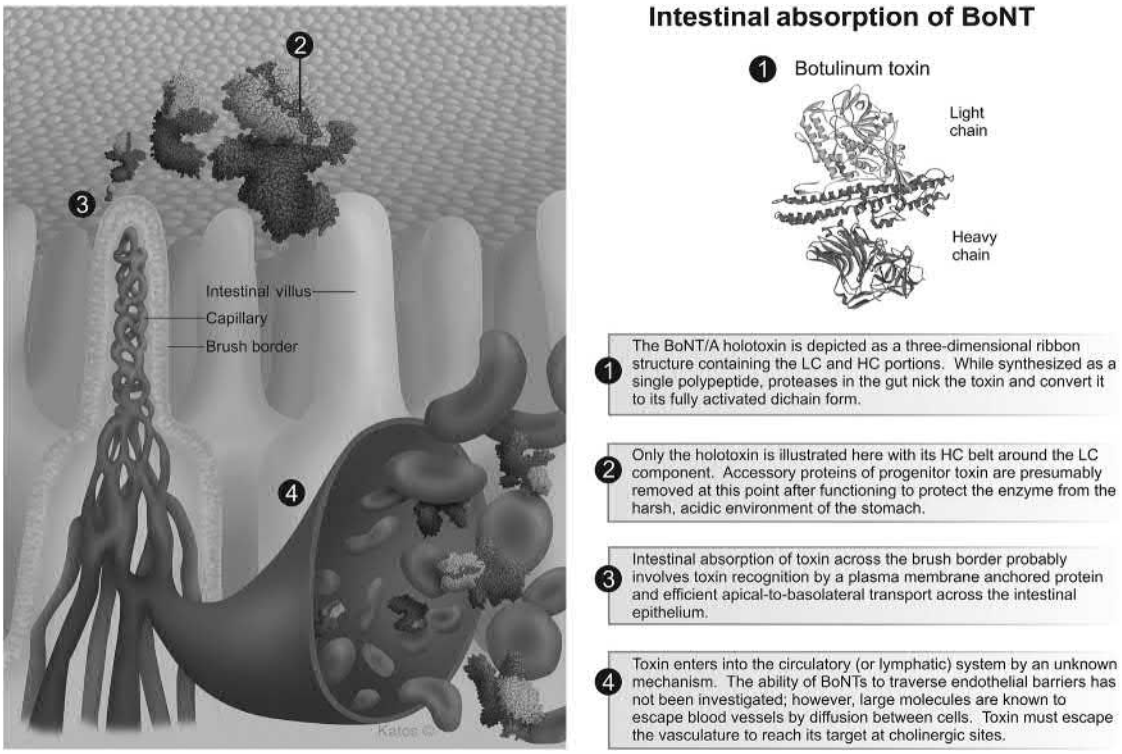

FIGURE 29.2 Intestinal absorption of BoNT. Neurotoxin present in the gut lumen as a result of foodborne (ingested toxin) or infant botulism (toxin synthesized by clostridial spores) must cross the epithelial membrane to reach the circulatory and lymphatic systems. The toxin presumably binds to an as-yet-unidentified receptor on the intestinal villus epithelium that is linked to an efficient transport process. Progenitor toxin contains nontoxic HA and NTNH accessory proteins, which are probably shed from the protein prior to entry across the intestine brush border. Progenitor toxin is thought to be too large for any significant rate of paracellular diffusion (Simpson, 2004). The ability of botulinum holotoxins without accessory proteins to traverse endothelial barriers has not been investigated; however, large molecules are known to escape blood vessels by diffusion between cells. Toxin escapes the circulatory system and reaches peripheral (and possibly central) cholinergic sites. These include NMJs, ganglia of the sympathetic and parasympathetic nervous system, postganglionic parasympathetic sites, and postganglionic sympathetic sites that release ACh. *Illustrations are copyright-protected and printed with permission from Alexandre M. Katos.*

Dolly, 1986; Blasi et al., 1993; Montecucco et al., 1994; Schiavo et al., 1995). BoNT thereby prevents docking of SVs with presynaptic plasma membrane by selective proteolysis of synaptic proteins. Each stage in BoNT action provides a potential point for pharmacological intervention.

29.5.2 Toxin stability

29.5.2.1 Biological stability of the toxins in the gastrointestinal tract

A major factor to consider in botulism is the stability of both the organism and the toxin. A variety of factors can affect the stability of ingested BoNTs within the gastrointestinal tract. The oral potency of the toxins is closely related to their ability to withstand the conditions found in these biological compartments prior to absorption into the lymphatics and general circulation. The stability of BoNT preparations, therefore, has been examined in the gastrointestinal compartments of intoxicated animals, as well as under different enzymatic and acidic conditions in vitro. Some of the earliest work on BoNT intoxication indicated that the stability and resulting potency of type A toxin vary both qualitatively and quantitatively in different rodent species (Minervin and Morgunov, 1941).

Several groups have evaluated the influence of ingested foods on the gastrointestinal stability and potency of BoNTs. Lamanna and Meyers (1959) reported that the ingestion of protein- or fat-containing foods prior to oral type A exposure in mice resulted in a moderate increase in toxicity. The mechanisms by which food intake enhanced toxin potency were not clarified; however, the relatively small observed increases (twofold) could have been due to normal experimental variation in determining oral toxicity values. The same study demonstrated that fluorescein-labeled type A toxin was quickly destroyed in the stomachs of mice. Crystalline and purified toxins form stable complexes with albumin and other proteins found in food and serum (Lamanna and Meyers, 1959). Albumin was later shown to prevent loss of potency when type A toxin was exposed to a wide range

of pH values (Zacks and Sheff, 1967). This observation was consistent with the more recent finding that the enzymatic activity of BoNT/A was enhanced in the presence of albumin (Schmidt and Bostian, 1997).

Subsequent studies expanded upon this early work by investigating the stability of other toxin types and preparations in solutions having different pH values and other conditions similar to those encountered in the gastrointestinal tract. Type C1 toxin was stable in most acidic and basic environments, as significant inactivation (as indicated by loss of toxicity in a mouse lethality assay) was observed only following exposure to extreme pH values (i.e., pH 1.8 and 12) (Halouzka and Hubalek, 1992). Progenitor type E toxin was more stable than its derivative (purified) form, which was subject to rapid inactivation when exposed to pH values less than 4.0 (Sakaguchi and Sakaguchi, 1974). This study demonstrated that type E progenitor toxin dissociated either during or after gastrointestinal absorption in mice, as only the derivative component could be detected in the blood and lymph following oral administration of the progenitor form.

Similar findings on the relatively high stability of progenitor versus derivative forms of the other toxin serotypes have been reported in other studies. Types A, B, and F progenitor toxins were more stable under conditions of low pH, as well as more resistant to digestion by pepsin and papain, than their corresponding derivative toxins (Sugii et al., 1977a,b).

The derivative forms of toxins A and B were almost completely inactivated after 10 min of peptic digestion at pH 2.0, while the progenitor forms retained over 60% toxicity after an 80-min treatment (Sugii et al., 1977a). Crystalline type A toxin was shown to be partially resistant to proteolysis by trypsin, retaining 25% of the potency of control-treated toxin even after a 72-h trypsin digestion at 37°C (Coleman, 1954). The crystalline toxin was more readily inactivated by digestion with pepsin at pH 1.4 and chymotrypsin at pH 6.5. Interestingly, another group reported that the potency of type A toxin was weakened by 80% after a 5-h incubation in phosphate buffer (pH 7.5), while toxins C1 and D maintained 100% toxicity under the same conditions (Miyazaki and Sakaguchi, 1978). These findings demonstrate both serotype- and enzyme-dependent effects on the in vitro stability of the BoNTs that are also likely to affect their persistence in the gastrointestinal tract. A similar pattern of stability among the various BoNT forms in gastrointestinal juices isolated from different animal species has been demonstrated (Sugii et al., 1977a). The progenitor forms for all toxin serotypes retained significant toxicity in comparison to their derived holotoxin counterparts.

Epitope mapping experiments suggested that the nontoxic component of the intact progenitor toxin complex covers a large portion of the binding domain of the neurotoxin (Chen et al., 1998). Toxin interaction studies also revealed that the purified neurotoxin adheres to lipid monolayers, while the progenitor complex is not subject to significant adsorption to the same monolayers. This observation led to speculation that the protective nontoxic components (HA and NTNH) may also facilitate progenitor toxin transit through the gastrointestinal tract by minimizing neurotoxin adherence to lipid membranes (Chen et al., 1998). On the other hand, toxicity studies suggest that adherence to lipid membranes is not critical for neurotoxin absorption since the intact progenitor complex is generally much more potent by the oral route than the purified neurotoxin. Moreover, the neurotoxin has been shown to protect the agglutination capacity of the associated nontoxic HA components within the progenitor toxin complex (Chen et al., 1998).

Importantly, the nontoxic HA components of the progenitor complex appear to protect the neurotoxin from proteolysis and degradation under pH extremes while the agglutinating activity of the nontoxic component is maintained by the presence of the neurotoxin. The type A progenitor toxin complex contains several HA components that might contribute to protecting the neurotoxin from degradation. One of these HA components, referred to here as *HA-33* (or *HA1* in some studies), was shown to interact directly with type A neurotoxin and to significantly increase its resistance to enzymatic proteolysis in vitro (Sharma and Singh, 2004). The authors of this work hypothesized that HA-33 provides protection against enzymatic degradation either by blocking the accessibility of protease-sensitive sites on BoNTs or by inducing structural changes within the neurotoxin itself.

Collectively, these studies offer important insight into the relative stability of the BoNTs within the gastrointestinal tract based upon their resistance to inactivation under various enzymatic conditions. In general, toxin stability directly correlates with the presence of the accessory HA and NTNH components of the multimeric progenitor toxin complex. These proteins appear to function in protecting the neurotoxin from degradation or inactivation. The various toxin serotypes also display unique resistances to enzymatic digestion although the basis for these differences is not known.

29.5.3 Oral intoxication: toxin absorption from the gastrointestinal tract

29.5.3.1 Role of progenitor toxin accessory proteins

The role of the nontoxic accessory proteins within the progenitor toxin complexes is not fully understood. They appear to function in protecting the ingested toxins from degradation and in facilitating absorption from the

gastrointestinal tract. Functional characterization of the HA and NTNH proteins has been advanced by biochemical techniques for generating toxin preparations containing only select components of the progenitor complex. These 7S toxins are relatively sensitive to proteolytic degradation and denaturation in the stomach (Schiavo et al., 1992). The auxiliary HA and NTNH proteins within the multimeric complex have been shown to dramatically increase the stability of the associated neurotoxin during transit through the gastrointestinal tract (Ohishi et al., 1977; Sugii et al., 1977a,b,c). The multimeric complex is then thought to readily dissociate either in the intestine or after absorption into the circulation. Most studies suggest that the accessory proteins do not appear to have any involvement in the activity of the toxins at peripheral nerve terminals. Thus, the HA and NTNH components are likely to be dispensable in disease pathogenesis after parenteral or respiratory exposure, where the toxins bypass the harsh conditions of the gastrointestinal tract.

29.5.3.2 Role of enterocytes

Both absorptive enterocytes and Peyer's patch-associated M cells have been implicated in toxin transcytosis from the gastrointestinal tract after oral exposure. Peyer's patches are collections of lymphoid tissue that are part of the gut-associated lymphoid tissue. M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. Park and Simpson (2003) indicate that knockout mice deficient in Peyer's patch-associated M-cell complexes are still susceptible to both oral and respiratory botulinum intoxication and the development of HC-specific antibody responses. Based upon these results, the authors suggest that M cells are not likely to be involved in toxin uptake and processing from the respiratory tract. In addition, both cell types have comparable transcytosis rates (M cells are five times as efficient in transcytosis than intestinal enterocytes), but enterocytes greatly outnumber M cells; therefore, gastrointestinal enterocytes are the predominant cell types involved in toxin uptake and processing from the gastrointestinal tract.

Maksymowych and Simpson (1998) used transwell culture systems with various transformed epithelial cell lines to evaluate the fate of the HA components of type A progenitor toxin complex. Radiolabeled preparations of both BoNT/A and HA were taken up by cultured T-84 human colon carcinoma cells by bulk endocytosis. However, efficient delivery across the T-84 cells was observed only for the neurotoxin.

29.5.4 Respiratory intoxication

The potential threat posed by aerosolized botulinum toxins is emphasized by their ease of production, their extremely high potency relative to other biological toxins, and their use in various weaponization programs over the past several decades (Arnon et al., 2001). This threat, along with the relative lack of information on respiratory toxicity and pathogenesis in humans, has fueled research on inhalational botulism in several animal models, including mice, rabbits, guinea pigs, mongrel dogs, and rhesus monkeys.

29.5.4.1 Toxin absorption from the respiratory tract

The relative persistence and absorption of the toxins following experimental respiratory exposure have been investigated in a few animal species. An early literature review suggests that type A toxin is more potent in mice by the respiratory route than by subcutaneous (s.c.) administration but less potent by the intraperitoneal (i.p.) route (Morton, 1961).

Guinea pigs were shown to be highly sensitive to inhaled botulinum toxins when compared to other rodent species. Respiratory penetration and retention of inhaled toxin are higher in guinea pigs than mice (Lamanna, 1961). Toxin could be detected in the lungs of guinea pigs after intranasal (i.n.) administration of only two mouse lethal doses of type E toxin, although detection in the blood or liver required higher doses (Sergeyeva, 1962, 1966). Guinea pigs were also reportedly more susceptible than mice to type A toxin by inhalation because shorter incubation periods were observed in guinea pigs prior to the onset of acute disease (Iakovlev, 1958).

Although inhalational botulinum intoxication was investigated in other animal species, these studies have not provided specific data on toxin absorption. The behavior of BoNTs in the respiratory tract was only recently investigated. Park and Simpson (2003) studied the properties of pure BoNT/A neurotoxin both in vivo and in vitro using mice and pulmonary cell culture models, respectively. Mean survival times were compared in mice receiving various doses of pure BoNT/A either i.n. or i.p. Pure BoNT/A was found to be a potent i.n. poison, although the toxicity (as determined by mean survival time) associated with i.p. administration was somewhat higher. Mean survival times in mice were less than 100 (i.p.) or 600 min (i.n.) after administration of 0.1 μg pure toxin; 75 (i.p.) or 400 min (i.n.) for 1 μg toxin; and 120 min (i.n.) for 10 μg toxin (Park and Simpson, 2003). As seen with oral and parenteral routes, a linear relationship existed between the log of the intranasal dose administered and the geometric mean survival time. The HA and NTNH component of the progenitor toxin did not enhance toxicity, establishing different requirements for the stability and absorption of inhaled versus ingested toxin (Park and Simpson, 2003).

Transwell experiments were also performed to investigate BoNT/A transcytosis across a human pulmonary

adenocarcinoma cell line (Calu-3), the MDCK cell line, and a primary rat alveolar epithelial cell line (Park and Simpson, 2003). Efficient BoNT/A transcytosis in both directions across polarized Calu-3 monolayers was observed, while toxin transcytosis occurred at a much lower rate across MDCK cells. These findings were in agreement with previous work demonstrating that the efficiency of BoNT/A transcytosis across MDCK monolayers was much lower than that observed across gut epithelial cells (Maksymowych and Simpson, 1998). BoNT/A transcytosis was also observed across primary rat alveolar cells, although at a slightly slower rate than that seen for the human adenocarcinoma cells (Park and Simpson, 2003). While the LC of BoNT/A was not essential for transcytosis, HC apical−basolateral (A−B) and basolateral−apical (B−A) transcytosis rates were somewhat lower than those of intact BoNT/A for both Calu-3 cells (HC 53% lower than BoNT/A for A→B; 45% lower for B→A) and rat alveolar cells (HC 62% lower for A→B; 17% lower for B→A). The transcytosis process was shown to involve an active-energy-dependent mechanism and was significantly inhibited by toxin preincubation with immune serum (Park and Simpson, 2003).

An important caveat to consider when evaluating the relevance of these in vitro studies is the use of pulmonary adenocarcinoma and alveolar epithelial cell lines in modeling respiratory absorption. It is generally believed that systemic absorption of inhaled particles is more likely to occur within the distal regions of the respiratory tract; therefore, particles must pass through thinner membranes in the deep lung and are less susceptible to nonabsorptive particle clearance (Lamanna, 1961). Some potential also exists for significant particle absorption from the nasopharyngeal and tracheobronchial regions of the respiratory tract; the cell lines utilized in these in vitro studies clearly do not account for this absorption potential. Importantly, investigators in the field have recently sought to characterize the specific cell types involved in toxin absorption from the respiratory tract.

M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. The studies of Park and Simpson (2003) indicate that M cells are not the major players in transepithelial transport of toxin across the respiratory epithelium. Additional studies directly investigating the absorption of inhaled BoNTs do not exist.

29.5.5 Toxin binding and uptake into target tissues

The remaining steps of BoNT pathogenesis following neurotoxin absorption are thought to be similar for both the respiratory and gastrointestinal exposure routes. After oral or inhalational exposure, the neurotoxins are absorbed from the gut or respiratory tract, respectively, into the lymphatics and circulation for transport to peripheral cholinergic synapses (Simpson, 2004). Fig. 29.3 illustrates the NMJ, a major target for the actions of BoNTs. BoNTs are taken up presynaptically at the endplate region of NMJs (Verderio et al., 1999) and at other cholinergic synapses. Toxin binding involves high-affinity presynaptic receptors. These receptors have recently been identified as a combination of polysialogangliosides, SV protein 2 (SV2), and synaptotagmin (Verderio et al., 2006). Each serotype displays an affinity for a unique combination of receptors. For example, BoNT/B recognizes synaptotagmin II (and I) and ganglioside lipids (Dong et al., 2003) (see Fig. 29.3).

BoNT/A involves recognition of SV2C, SV2A, and SV2B (Dong et al., 2003); binding to SV2C also involves a lipid. After toxin binding, the complex is internalized by what is believed to be a clathrin-mediated endocytotic process.

29.6 Toxicokinetics

The onset of symptoms in botulism depends upon the amount of toxin ingested or inhaled and the related kinetics of absorption. Time to onset can range from as short as 2 h to as long as 8 days, although symptoms typically appear between 12 and 72 h after consumption of toxin-contaminated food (Lecour et al., 1988; Arnon et al., 2001). In a review of 13 foodborne botulism outbreaks involving 50 patients from 1970 to 1984, the incubation period ranged from 10 h to 6 days (Lecour et al., 1988).

29.6.1 Foodborne toxicity

29.6.1.1 Toxin persistence in the circulation and transit to target tissues

Case reports of human foodborne botulism incidents offer some information on toxin persistence and transit after oral exposure in humans. Koenig et al. (1964) reported that circulating toxin was detected in five out of six patients suffering from type E botulism after consuming contaminated fish by the mouse lethality assay on serum samples collected from the patients from 1 to 10 days after foodborne exposure. Serum from one of the patients who rapidly succumbed to disease contained approximately 8 $MIPLD_{50}$/mL; extrapolation of this value yields an estimate that 20,000−24,000 human LD_{50}s were in this individual's circulation (Koenig et al., 1964). The toxin isolated from the serum of these clinically ill patients was not further activated in vitro by trypsin treatment. This observation was in agreement with other studies demonstrating cleavage of the single-chain prototoxin

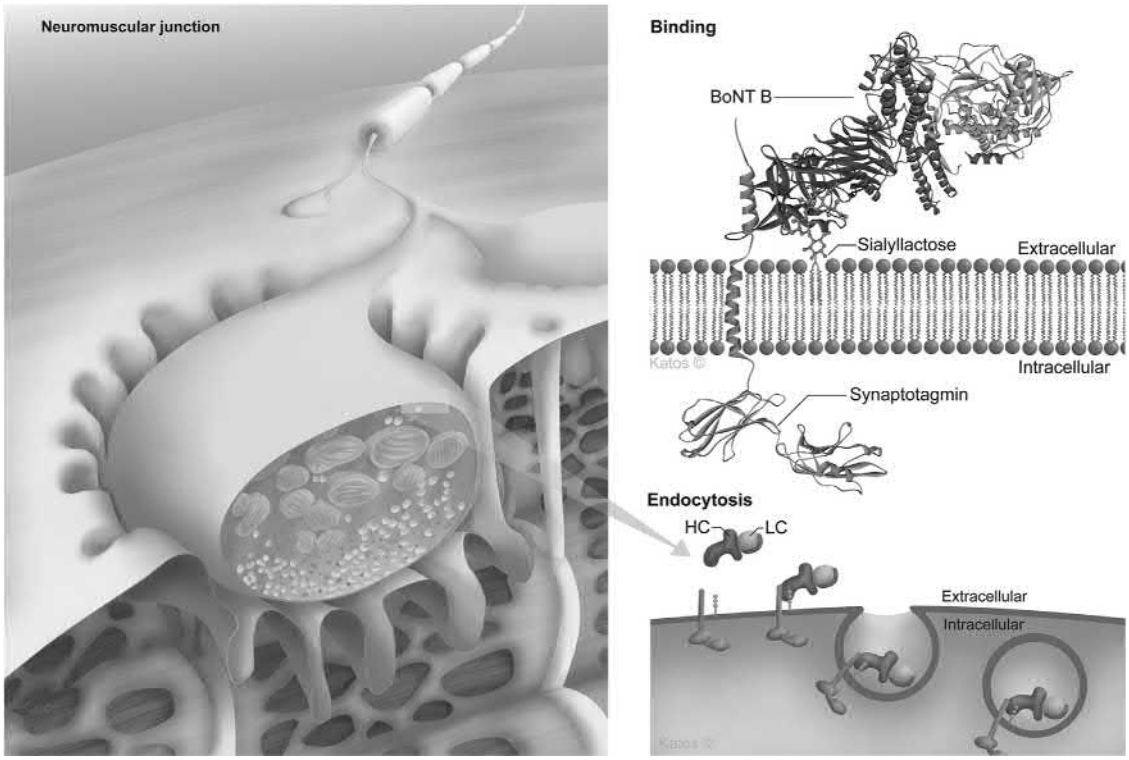

FIGURE 29.3 Toxin binding and internalization at the NMJ. (Left panel) A mammalian NMJ is illustrated with the alpha motor neuron innervating skeletal muscle at specialized junctional folds in the membrane (Couteaux and Bourne, 1973). Invaginations of the T system are also illustrated at the level of transition between the A and I bands. The axon loses its myelin sheath and dilates to establish irregular contact with the muscle fiber. Muscle contraction begins with the release of ACh from SVs (tiny spheres) at the motor endplate region. ACh binds to postsynaptic, muscle-type nicotinic ACh receptors and causes an increase in the permeability of the sarcolemma. This process is propagated to the rest of the sarcolemma and ultimately to the sarcoplasmic reticulum (SR) by the T system. An increase in SR permeability liberates calcium ion (Ca^{2+}) stores, resulting in the sliding of illustrated muscle filaments and muscle contraction. BoNTs bind and internalize at the presynaptic side of the NMJ (Verderio et al., 1999). Release of BoNTs into the cytosol results in inhibition of ACh release and flaccid paralysis of innervated muscle. [Top-right panel (toxin binding)] A three-dimensional ribbon structure of BoNT/B is illustrated. The receptor-binding domain of BoNT/B HC binds to synaptotagmin (Syt-II) and ganglioside (sialyllactose) receptors of the presynaptic motor endplate. Each BoNT serotype binds to a different set of receptors in the membrane (Verderio et al., 2006). [Bottom-right panel (endocytosis)] Receptor-mediated endocytosis of BoNT/B holotoxin is illustrated in this panel. The remaining steps in BoNT toxicity involve acidification of the endosome, pH-induced conformational change in the toxin, translocation of BoNT LC across the endosomal membrane with the aid of BoNT HC, and proteolytic degradation of target SNARE proteins by LC. *Illustrations are copyright-protected and printed with permission from Alexandre M. Katos.*

to the active dichain form within the gastrointestinal tract. Importantly, circulating toxin was not detected in a patient with minimal disease 11 days after ingestion of contaminated food (Koenig et al., 1964). Six out of seven individuals who had consumed the contaminated fish but did not develop clinical illness also lacked circulating toxin. Circulating toxin, therefore, was detected much more consistently in symptomatic patients associated with this outbreak than in subjects who were unaffected after toxin ingestion (Koenig et al., 1964).

Koenig et al. (1964) also reviewed previously published literature on the detection of circulating toxin in botulism patients. Circulating toxin (primarily serotype B) had been detected in select patients from 2 to 25 days after consumption of contaminated food and was rarely detected in type A botulism patients (Koenig et al., 1964). The authors suggested that serotype-specific differences in the persistence of circulating toxin might be attributed to their unique avidities to target tissues. Circulating toxin is generally detected only at very low levels at or immediately prior to death in lethally intoxicated patients (Ono et al., 1970).

Efforts have been made to determine the kinetics of the accessory components of the progenitor toxin complexes after systemic absorption of BoNTs. Iida et al. (1970) found that circulating type E toxin was shown to exist in the 7S form after oral administration of the progenitor toxin to rabbits, suggesting that the larger toxin complex dissociated at some point during or after absorption from the gastrointestinal tract. Similar findings were reported on the absorption and persistence of progenitor type A toxin in the rat; the mean sedimentation value of

toxin in the lymph after intraduodenal instillation was 7.9S, significantly lower than that of the crystalline toxin (Heckly et al., 1960).

29.6.2 Inhalation toxicity

29.6.2.1 Toxin persistence in the circulation and transit to target tissues

Very limited data are available on the persistence of BoNTs in the circulation following inhalation exposure in any animal species. These data indicate that circulating toxin can be detected soon after exposure but is subsequently cleared rapidly from the circulation. Park and Simpson (2003) reported on the time course for appearance (and amount) of either purified BoNT/A or type A HC in the circulation of mice after intranasal exposure. Maximum serum levels were observed at 2 h postexposure for both proteins, although the peak values were higher for BoNT/A than for HC. Rapid clearance was observed over the next few hours.

An earlier study showed that type A toxin could be detected primarily in the lungs and liver rather than the serum of guinea pigs after i.n. exposure (Sergeyeva, 1962). The same group reported on the correlation between administered toxin dose and detection of toxin in the blood, lungs, and liver of guinea pigs intoxicated via the i.n. route (Sergeyeva, 1966). Type E toxin was detected in the lungs of guinea pigs after i.n. administration of two lethal doses, while toxin appeared in the blood or liver only following i.n. administration of at least five lethal doses. The organ distribution patterns were similar in guinea pigs after inhalation exposure to types A, B, or C toxins (Sergeyeva, 1966). These studies did not address the potential for persistent toxin detection in the lymph after inhalational intoxication, despite the fact that other routes of exposure result in significant absorption into the lymphatics.

While scant literature is available on persistence and distribution after inhalation exposure, several studies have evaluated the systemic behavior of parenterally administered toxins. One group investigated toxin persistence in serum and tissue distribution in white mice following i.v. administration of 1000 lethal doses of ^{35}S-labeled type B toxin (Pak and Bulatova, 1962). Mice were sacrificed at 20, 60, and 150 min after toxin administration, and blood and tissues were harvested for toxin distribution analysis. These mice showed symptoms of severe intoxication, including atypical breathing patterns and paralysis, at 150 min postexposure. Toxin levels (as determined by radioactivity) were highest in the lung 20 min after toxin injection, followed by the liver, heart, kidneys, intestines, and brain (Pak and Bulatova, 1962). Radioactivity levels in the blood, as well as the liver, heart, intestines, and brain, were further reduced after 60 min posttoxin injection. Serum toxin concentrations were lower than those detected in any other tissue at all times (Pak and Bulatova, 1962). The authors concluded that the toxin rapidly escaped from blood to various other tissues, suggesting the capacity for unimpeded passage of the toxin through the vasculature and cellular membranes.

Somewhat slower kinetics for toxin clearance from the circulation were observed in dogs following parenteral [i.v., i.p., or intramuscular (i.m.)] exposure to type A toxin (House et al., 1964). Serum toxin persistence was evaluated in mongrel dogs receiving 8000−10,000 mouse units/kg of type A toxin. Peak serum toxin levels were detected 5 h after i.p. administration (13% of injected dose), 12 h after i.m. administration (9% of the injected dose), and within only 3 min after i.v. administration (79% of the injected dose) (House et al., 1964). The relative clearance kinetics were slower after i.m. and i.p. exposure than for i.v. administration, as serum toxin levels were identical 22 h after injection via all three rounds (approximately 6% of the injected dose). Some serum toxin activity could be detected by the mouse lethality assay for 2−4 days after parenteral administration. Serum toxin patterns were also evaluated in rhesus monkeys following i.v. administration of type A toxin (Stookey et al., 1965). Serum toxin levels dropped by about 50% of maximum within 16−24 h after i.v. injection, and previous exposure did not affect toxin clearance rates after the administration of subsequent doses.

Another study investigated circulating toxin levels in weanling pigs (5−12 weeks old) following parenteral administration of toxin types A, B, C1, and D (Smith et al., 1971). Toxin was cleared from the circulation less than 24 h after i.v. injection of type B (560 $MIPLD_{50}$/kg), type C1 (5,000 $MIPLD_{50}$/kg), or type D (60,000 $MIPLD_{50}$/kg) toxin. In contrast, toxin could consistently be detected in the serum over the entire 4-day period prior to death in pigs injected with serotype A (21,400 $MIPLD_{50}$/kg) (Smith et al., 1971). Serum toxin levels were 100 MIP_{50}/mL at 24 and 48 h after injection of type A toxin, 30 $MIPLD_{50}$/mL after 3 days, and 10 $MIPLD_{50}$/mL after 4 days. These findings indicated serotype-specific differences in the persistence of circulating BoNTs, at least in systemically intoxicated pigs.

Although these studies provide some insight into the persistence of circulating toxin after parenteral administration, they do not necessarily reflect the behavior of absorbed toxin after respiratory exposure. The route of administration may not have a significant impact on the behavior of toxin once absorbed into the serum and lymph, but the patterns and kinetics of absorption into the circulation might be quite different after inhalational versus parenteral exposure. Respiratory exposure could lead to a different proportion of toxin taken up into the

circulation, lymphatics, or both over a given time period than that seen after systemic injection. Such discrepancies might affect both the quantitative persistence of circulating toxin and its transit to peripheral target tissues. At this point, information is not yet available on the homing and distribution of toxins to target nerve tissues. The available literature also provides no insight on the mechanisms by which toxin is removed from the circulation, either through extravasation and uptake in target tissues or by metabolic processes. In the future, such data will be important in characterizing the pathogenesis of botulism after respiratory intoxication and other routes of exposure.

29.7 Mechanism of action

ACh release from presynaptic vesicles depends upon a propagated action potential, localized depolarization at the presynaptic motor endplate, proper SNARE complex formation (i.e., SNAP-25, syntaxin, and synaptobrevin), and SV docking with the presynaptic membrane (see neuromuscular transmission in the absence of BoNT; Fig. 29.4). Regardless of the exposure route, BoNTs lead to inhibition in the release of ACh from peripheral cholinergic nerve terminals resulting in flaccid paralysis (Simpson, 1986; see Fig. 29.5). The specific target for BoNT/A and /E is the 25-kDa vesicle-docking protein SNAP-25; BoNT/A cleaves the last nine residues, whereas BoNT/E cleaves a larger 26-residue fragment from the C-terminus of this protein (Blasi et al., 1993; Montecucco et al., 1994). The target of BoNT/B is the small transmembrane protein synaptobrevin/VAMP located on the surface of small SVs (Schiavo et al., 1995). The enzymatically active portion of the 150-kDa BoNT is the 50-kDa LC; the role of the 100-kDa HC involves binding to cholinergic nerve endings and intracellular penetration via receptor-mediated endocytosis (Simpson, 1986, 2004; Montecucco et al., 1994).

29.7.1 Heavy chain

The HC of the BoNTs has been shown to mediate toxin binding and internalization at cholinergic nerve terminals (Daniels-Holgate and Dolly, 1996; Simpson, 2004). The mostly β-strand-containing carboxy-terminus of the HC appears to be directly involved in toxin binding, while the mostly α-helical amino-terminal region mediates

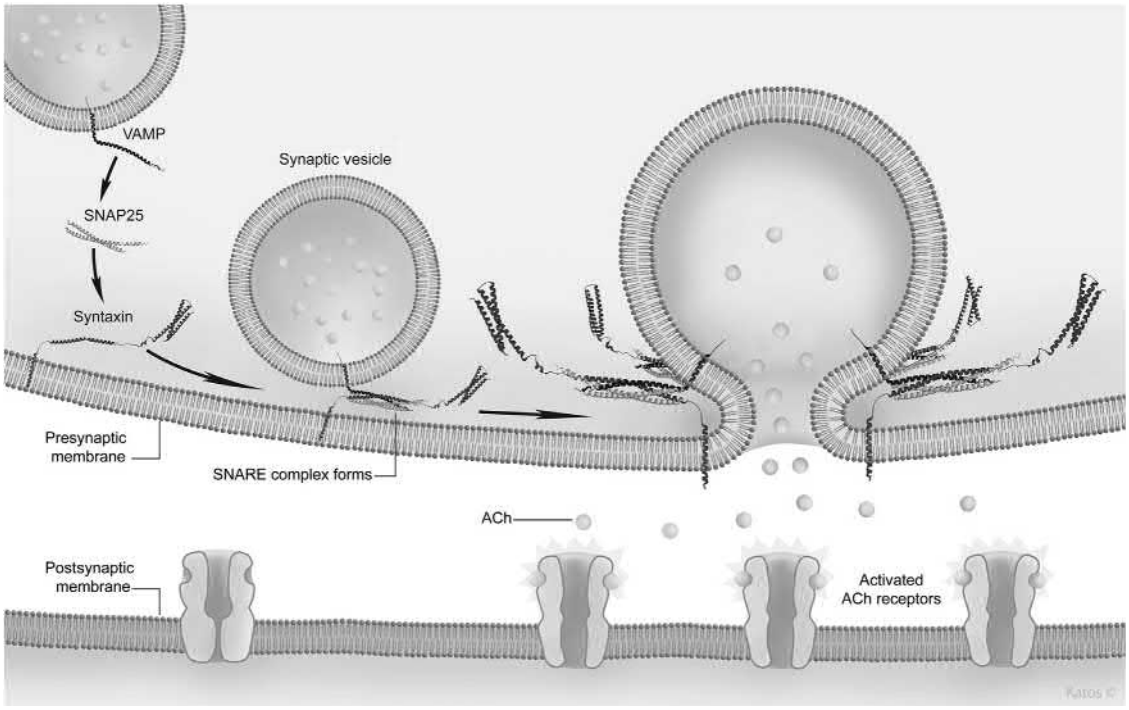

FIGURE 29.4 Neuromuscular transmission in the absence of BoNT. A nerve impulse is transmitted to the effector (muscle) cell by neurotransmitter liberated at the synapse. When the action potential arrives at the axonal terminus to depolarize the presynaptic membrane, Ca^{2+} ions enter through voltage-dependent Ca^{2+} channels. Ca^{2+} ions facilitate the fusion of SVs, containing the neurotransmitter ACh, with the presynaptic membrane. Three SNARE proteins (syntaxin, synaptobrevin, and SNAP-25) are critical for SV fusion. So long as the SNARE complex is intact, ACh releases by exocytosis, diffuses across the synaptic cleft, and binds to postsynaptic muscle-type nicotinic ACh receptors. Binding of ACh makes the sarcolemma of the muscle cell more permeable to sodium, which results in membrane depolarization. Excess ACh is hydrolyzed by the enzyme cholinesterase bound to the synaptic cleft basal lamina. ACh breakdown is necessary to avoid prolonged activation of ACh receptors. *Illustrations are copyright-protected and printed with permission from Alexandre M. Katos.*

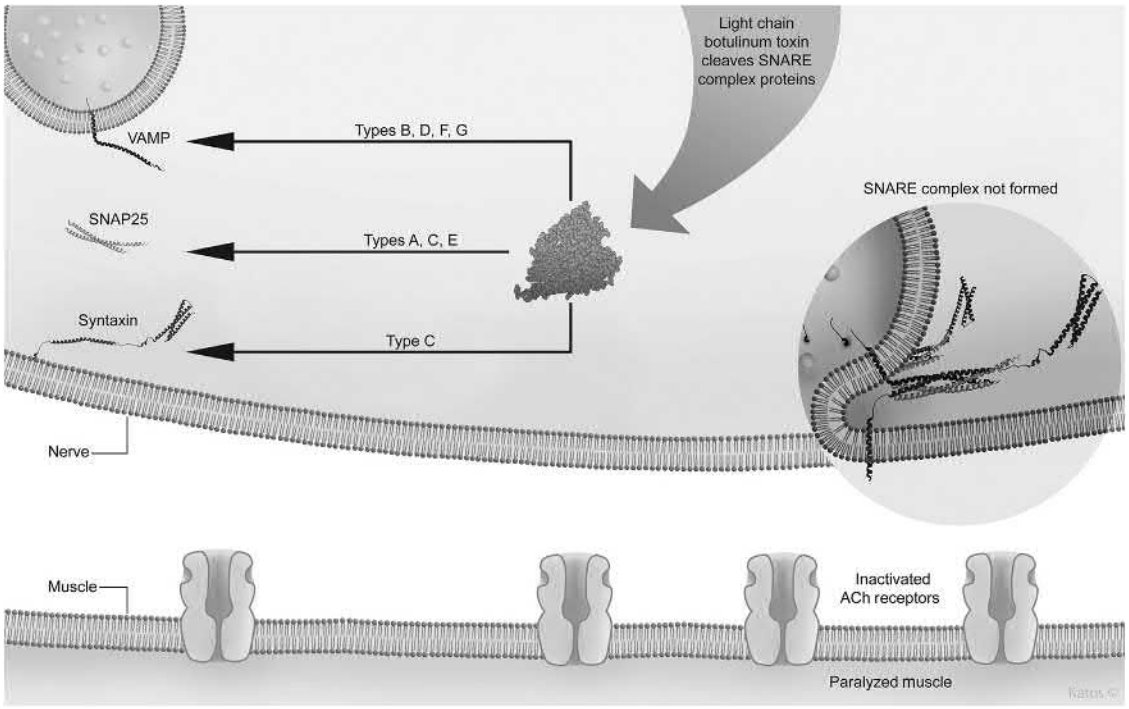

FIGURE 29.5 Neuromuscular transmission in the presence of BoNT. The catalytic LCs of the various serotypes cleave specific SNARE proteins. The SNARE complex does not form if any of the proteins are cleaved. In the presence of BoNT LC inside the axonal terminus, SVs will not fuse with the presynaptic membrane, ACh will not be released, and the muscle will not contract, resulting in paralysis at the NMJ. *Illustrations are copyright-protected and printed with permission from Alexandre M. Katos.*

translocation across the endosomal membrane (Simpson, 2004). Through mechanisms that have yet to be fully characterized, the toxins gain entry into the nerve terminal through receptor-mediated endocytosis followed by pH-induced translocation from the endosome to the cytosol. The ability of the Hn region to form transmembrane ion channels raises the possibility that they are intimately involved in translocating the toxic moiety into the cytoplasm (Koriazova and Montal, 2003). Once translocated into the cytosol, the toxic fragments exert their paralytic effects by inhibiting ACh release from NMJs as well as other peripheral cholinergic sites, including sympathetic and parasympathetic ganglia and postganglionic parasympathetic synapses (Lamanna, 1959; Simpson, 2004).

29.7.2 Light chain

These paralytic effects have been attributed to the proteolytic activity of BoNT LC on protein substrates required for vesicular exocytosis. BoNT LC inhibits neurotransmitter exocytosis through its zinc-dependent endoproteolytic activity. The LCs of the various neurotoxin serotypes possess distinct molecular targets within the peripheral cholinergic nerve terminals (Schiavo et al., 1992, 1993a,b, 1995). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects.

The intracellular proteins SNAP-25, syntaxin, and synaptobrevin (or VAMPs) normally interact with each other in mediating neurotransmitter release from cholinergic and other nerve terminals (see Fig. 29.4). Toxin types B, D, F, and G cleave the VAMPs, while types A, C1, and E act on SNAP-25; type C1 toxin also cleaves syntaxin (Dong et al., 2003). The functions of the various neurotoxins are even more specialized, in that one toxin type can cleave its substrate at a different site than that targeted by other toxin serotypes. For example, BoNT/A cleaves SNAP-25 between residues 197 and 198, resulting in the loss of 9 amino acids, while BoNT/E cleaves the same protein between residues 180 and 181, thereby removing 26 amino acids (Schiavo et al., 1993a).

Although the LCs of both BoNT/A and /E target SNAP-25, these two serotypes exert significantly different potencies and paralytic profiles in cultured neurons and in vivo. A potential molecular basis for this discrepancy was established by the finding that these neurotoxins target different cleavage sites within the SNAP-25 protein. BoNT/A cleavage generates a 197-residue truncated protein (P197) by cleaving the last 9 amino acids from the C-terminus of SNAP-25 while BoNT/E cleavage produces a 180-residue species (P180) by removing the final 26 residues (Schiavo et al., 1993a). A series of studies by Keller et al. (1999) and Keller and Neale (2001) provided

additional insight into the molecular mechanisms associated with the potent and persistent action of type A neurotoxin relative to type E.

Serotype-specific cleavage events provide insights into the differential activities of the toxins at nerve terminals. In some cases, substrate cleavage studies also revealed important information regarding interspecies differences in the activity of certain toxins. BoNT/B was shown to block neuromuscular transmission by cleaving VAMP proteins between residues Q76 and F77 in humans and mice (Bakry et al., 1997). However, the rat VAMP1 (synaptobrevin) protein sequence differs at this critical cleavage site, in that the glutamine at position 76 is replaced by a valine, rendering the region more resistant to proteolysis by BoNT/B (Bakry et al., 1997; Verderio et al., 2006). On the other hand, rats and mice were shown to have similar susceptibilities (body weight adjusted) to i.m. injection of type A toxin; rats have also been shown to be much more resistant than mice to type F toxin (Kauffman et al., 1985).

The specific paralytic profiles associated with each of the BoNTs are typically attributed to their unique proteolytic activities within the nerve terminal. These activities are known to be mediated by the LC components of the various neurotoxins. The various nontoxic components within the multimeric progenitor toxin complexes have traditionally been considered accessory proteins that primarily function to increase neurotoxin stability and, in some cases, to facilitate absorption. Yet studies in the recent past have suggested a potential role for the HA constituents in enhancing the endopeptidase activity of the LC (Cai et al., 1999; Sharma and Singh, 2004). It is widely believed that pure BoNT/A requires either proteolytic nicking or chemical reduction for significant SNAP-25 cleavage activity. However, new evidence suggests that the type A progenitor toxin complex is apparently highly active even in nonreduced form (Cai et al., 1999). Further research is needed to substantiate this preliminary work and to establish a more detailed understanding of the prerequisites for LC proteolytic activity.

A recent study by Sharma and Singh (2004) provided additional support for the expanded roles of at least one neurotoxin-associated protein within the type A progenitor complex. The HA-33 component, representing 25% of the accessory protein content of progenitor neurotoxin, significantly increases the proteolytic activity of both BoNT/A and /E in vitro and in rat synaptosome preparations. The addition of HA-33 to nonreduced BoNT/A leads to a 21-fold increase in GST-SNAP-25 fusion protein cleavage activity in vitro and a 13-fold enhancement of endopeptidase activity in rat synaptosomes (Sharma and Singh, 2004). Similar enhancement of proteolytic activity was seen when HA-33 was added to BoNT/E both in vitro and in rat brain synaptosomes.

The enhancement of SNAP-25 cleavage activity by HA-33 in rat brain synaptosomes was taken as evidence that the neurotoxin and the accessory protein both gain entry to the nerve terminal (Sharma and Singh, 2004). The possibility that an accessory component of the progenitor toxin complex could exert direct effects on LC endopeptidase activity within the nerve terminal could have important implications with respect to neurotoxin function in vivo.

Two recent reports revealed additional layers of complexity regarding the mechanisms involved in the distinct durations of action associated with the different toxin serotypes. Fernández-Salas et al. (2004) investigated the subcellular localization of BoNT/A, /B, and /E LC-GFP fusion proteins following overexpression in several different mammalian cell lines. The LC/A fusion protein was shown to localize within discrete plasma membrane compartments in both neuronal (PC12) and nonneuronal (HEK293, HeLa, and HIT-T15) cell lines, while LC/B was detected throughout the cell and LC/E was primarily found within the cytosol.

29.8 Toxicity

29.8.1 Lethality

BoNTs are the most potent substances known to humans. A comparison of the lethal nature of BoNTs in relation to other toxic chemicals and substances discussed throughout this book is provided in Table 29.2. The toxicity associated with oral exposure of a given species to BoNTs is significantly lower than that resulting from parenteral administration (see Table 29.3).

The susceptibility of various animal species to parenteral intoxication does not provide adequate indication of their sensitivity to gastrointestinal exposure (Lamanna, 1961). The estimated human LD_{50} of approximately 1 ng/kg for parenteral botulinum intoxication is similar to that reported for most laboratory animals (Arnon et al., 1995; Middlebrook and Franz, 2000). In contrast, the relative susceptibilities of humans and other animal species to oral intoxication vary significantly (Morton, 1961). A clinical review of human botulism reported that the ingestion of as little as 0.05−0.1 μg of BoNT/A may be sufficient to cause death in humans (Cherington, 1998). Human lethal doses have also been extrapolated from primate studies, yielding an oral lethal dose of approximately 70 μg for crystalline type A toxin for a 70-kg human (Arnon et al., 2001). The lethal human respiratory dose is estimated to be 0.7−0.9 μg and the i.v. or i.m. dose is 0.05−0.15 μg (Middlebrook and Franz, 2000; Arnon et al., 2001).

TABLE 29.2 Comparison of toxic chemicals and substances.

Chemical or toxin	Mouse i.v. LD_{50}[a] (mg/kg)	Chemical or toxin	Mouse i.v. LD_{50}[a] (mg/kg)
Botulinum toxin	0.00001	Strychnine	0.41
Batrachotoxin	0.002	Potassium cyanide	2.60
Anthrax lethal toxin	0.003–0.005	Mustard[b]	3.30
Ricin	0.005	Aflatoxin	9.5
Tetrodotoxin	0.01	Heroin	21.8
Saxitoxin	0.01	CR[c]	37
VX	0.012	Marijuana[d]	42
Abrin	0.02	BZ[e]	46
GD	0.066	CS[f]	48
GB	0.10	Caffeine[g]	62
GA	0.15	CN	81
TCDD[h]	0.182	Thujone[i] (absinthe)	134.2
Capsaicin	0.40	PAVA[j]	224

[a] *Intravenous dose that is lethal to 50% of mice.*
[b] *Mustard gas, 1,1′-thiobis[2-chloroethane].*
[c] *Riot control agent [dibenz-(b,f)-1,4-oxazepine].*
[d] *Delta-3,4-*trans*-tetrahydrocannabinol.*
[e] *Incapacitating agent, 3-quinuclidinyl benzilate.*
[f] *Riot control agent (o-chlorobenzylidene malononitrile).*
[g] *Riot control agent (chloroacetophenone).*
[h] *2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin (TCDD), a contaminant of the defoliant and herbicide Agent Orange.*
[i] *Constituent of wormwood absinthe, a popular emerald liquor, 4-methyl-1-(1 methylethyl) bicycle [3.1.0] hexan-3-one.*
[j] *Riot control agent (pelargonic acid vanillylamide).*

TABLE 29.3 Comparison of the lethalities of serotypes A–G by various routes of administration in the guinea pig.

Route of intoxication	Botulinum toxin serotype						
	A	B	C	D	E	F	G
Oral[a]	717	306	177	436	–	–	–
i.p.[b]	3.1[c]–5.2[d]	4.2[d]–6.5[c]	1.6[d]–3.2[c]	3.0[e]–6.4[c]	34.3[d]–78[e]	–	40–100[f]
i.m.[g]	4.3[c]	6.9[c]	3.1[c]	8.7[c]	102[c]	–	–
s.c.[h]	6[i]–30[j]	–	–	3[k]	100[l]	30–30[k]	–
Aerosol	141	350	87	186	778	–	–

[a] *The doses are normalized to mouse i.p. LD_{50} units.*
[b] *Intraperitoneal administration.*
[c] *Gelzleichter et al. (1998a,b).*
[d] *Cardella et al. (1963).*
[e] *Lamanna (1961).*
[f] *Cicarelli et al. (1977).*
[g] *Intramuscular administration.*
[h] *Subcutaneous administration.*
[i] *Morton (1961).*
[j] *Sergeyeva (1962).*
[k] *Dolman and Murakami (1961).*
[l] *Sergeyeva (1966).*

29.8.2 Oral toxicity

An earlier report suggested that humans are more susceptible than monkeys to type A toxin by the oral route based on toxin dose estimates in foodborne botulism case studies (Morton, 1961). This same study also summarized the susceptibility of numerous other animal species to oral botulinum intoxication. Mice, monkeys, and guinea pigs were considered highly susceptible, while chickens, rabbits, horses, dogs, rats, and cattle were classified as more resistant, and ferrets, mink, and hogs were deemed resistant. The oral lethal doses of type A toxin in guinea pigs (1000−3000 MIPMLD) and monkeys (2000 MIPMLD) (Morton, 1961) are similar to the estimated oral lethal dose for humans (7000 MIPMLD).

Morton (1961) provided evidence for an estimated human oral lethal dose of much less than 3500 MIPMLD for type B toxin. This estimate was based on an earlier report describing a fatal type B human botulism case resulting from the ingestion of 3500 MIPMLD in toxin-contaminated cheese (Meyer and Eddie, 1951). A man weighing 104 kg consumed approximately 70 g of contaminated cheese; repeated tests of the cheese indicated that it contained only 50 MLD/g of type B toxin. The patient first developed somewhat atypical disease symptoms of nausea, vomiting, diplopia, dysphagia, phagodynia, and instability within 7 h of exposure (Meyer and Eddie, 1951). The man was later hospitalized and developed symptoms more characteristic of foodborne botulism within 18−20 h. He died 57 h after toxin ingestion despite receiving 35,000 units each of both type A and B antitoxins (Meyer and Eddie, 1951). Therefore, it was determined that 3500 MIPMLD is much greater than the minimum lethal dose of type B toxin for humans due to the rapid onset of illness and severity of disease (Morton, 1961). The same study reported a woman who died from botulism 42 h after consuming a small piece of toxin-contaminated pear.

29.8.3 Inhalation toxicity

Naturally occurring botulism cases in humans and other animal species are almost exclusively associated with the ingestion of toxin- or spore-contaminated foods. The level of knowledge in the published literature on toxin absorption following inhalational exposure is, therefore, much more limited than that associated with gastrointestinal intoxication. The potencies of inhaled BoNTs have been investigated in several experimental animal species. In a review of the early literature on this topic, Morton (1961) reported comparatively similar ratios (5.9:1) of oral to respiratory toxicity (the comparative lethal doses for toxin administered via the oral versus the respiratory route) for type A toxin in guinea pigs and mice. Iakovlev (1958) concluded that guinea pigs were more susceptible than mice to type A toxin by inhalation because they succumbed to intoxication after a shorter incubation period (1−2 days, vs 3−4 days for mice). Several technical reports established more specific guinea pig inhalation toxicity data for serotypes A through E (Jemski, 1960, 1961b).

29.8.4 Clinical toxicity

The natural occurrence of human foodborne and infant botulism translates into a wealth of information on the clinical signs and symptoms of disease. This information can be compared to the array of physiological and pathological findings in various species of experimental animals after oral administration of BoNTs. The ability of inhaled BoNTs to produce illness has also been documented in humans and in several experimental species. The primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism; however, infants display a unique clinical picture of botulism. In addition, the various toxin serotypes are usually associated with analogous clinical presentations, with the most severe cases of foodborne botulism being caused by the ingestion of type A toxin.

Exposure to BoNT via oral or inhalational routes results in symptoms indicative of an inactive peripheral cholinergic system due to inhibition of ACh release from the nerve terminal. The time to onset of disease depends on the amount of toxin ingested and ranges from several hours to a few days after oral exposure (Lecour et al., 1988; Arnon et al., 2001). Prominent signs and symptoms of intoxication common to all serotypes and various routes of exposure include the following, in order of descending frequency: dysphagia, xerostomia, diplopia, dysarthria, fatigue, ptosis of the eyelids, constipation, arm weakness, leg weakness, gaze paralysis, blurred vision, diminished gag reflex, nausea, facial palsy, dyspnea, emesis, tongue weakness, sore throat, dizziness, dilated or fixed pupils, abdominal cramps, reduced or failed reflexes, nystagmus (involuntary rapid eye movement), diarrhea, ataxia, and paresthesia (reviewed by Arnon et al., 2001).

29.8.4.1 Foodborne botulism

Human foodborne botulism presents as an acute, symmetric, flaccid paralysis that generally involves multiple cranial nerve (CN) palsies initially, termed *bulbar involvement*. Early symptoms involve paralysis of the motor components of the CNs. The motor components are derived from cell bodies located in the brain, with axons that exit the cranium to control muscles, glandular tissue,

or specialized muscle in the heart and gastrointestinal tract. Paralysis by BoNTs leads to ptosis and dilated pupils (CN III); disconjugate gaze and blurred vision (CN III, IV, VI); facial droop or palsy (CN VII); dysphagia, dysarthria, and absence of gag reflex (CN IX, X); tongue weakness (CN XII); and weakness of neck strap muscles (CN XI). Botulism patients typically develop difficulty in seeing, speaking, or swallowing in the early phases of intoxication. As paralysis extends caudally, toxic signs and symptoms include loss of head control, hypotonia, generalized weakness, and flaccid paralysis or floppy appearance (infants and children). In infants and young children exposed to BoNT, floppy appearance and constipation may be the only presenting signs to warrant a diagnosis of BoNT exposure, since obtaining a reliable history may not be possible in this population.

Loss of the gag reflex and dysphagia may require intubation and mechanical ventilation. Deep tendon reflexes are often lost during later stages of intoxication, and death in untreated patients results from airway obstruction or inadequate tidal volume (Arnon et al., 2001). Respiratory failure is the most serious clinical manifestation of botulism, and the decline in mortality associated with foodborne botulism is primarily due to improvements in ventilatory support (Lecour et al., 1988). Around 60% of botulism patients in the United States require mechanical ventilation at some point during their hospitalization and treatment (Varma et al., 2004). In severe botulism cases (as in the previously mentioned case of the Florida physician involving research grade type A toxin instead of BOTOX for facial muscle paralysis), respiratory support may be required for prolonged periods of time, and autonomic dysfunction may persist for a period ranging from months to years (Mackle et al., 2001).

Other clinical forms of the disease share many of these signs and symptoms. The presentation and duration of the disease are coupled to the relative persistence of the toxin in blocking the release of ACh at peripheral nerve synapses. Although untreated botulism is potentially deadly, the availability of antiserum has dramatically reduced the mortality rates for the common clinical manifestations of the disease. Severe cases of foodborne botulism may still require ventilatory support for over a month, and neurological symptoms can sometimes persist for more than a year (Mackle et al., 2001).

29.8.4.2 Infant botulism

The characteristic symptoms of infant botulism are poor sucking, constipation, generalized weakness, floppy appearance, and respiratory insufficiency (Cox and Hinkle, 2002). Infant botulism may quickly progress to respiratory failure if not treated. The development of the intestinal flora has been demonstrated to suppress the germination and growth of *C. botulinum* spores in mice (Sugiyama and Mills, 1978). Ingestion of honey by infants is the classic scenario cited in infant botulism; therefore, honey is not recommended in this susceptible population (Arnon et al., 1998).

29.9 Risk assessment

BoNTs present a very real threat to the public health and are the most toxic substances known to humans. In a military or bioterrorist incident, intoxication by BoNT is likely to occur by inhalation of aerosolized toxin or by ingestion of contaminated food or beverages (Franz and Zajtchuk, 1997; Sobel et al., 2004). Although municipal water systems are considered to be safe from BoNT attacks, due to chlorination and dilution, it is not known whether current water treatments adequately decontaminate the toxin. Furthermore, bottled mineral water and milk (Sobel et al., 2004; Kalb et al., 2005) are obvious targets for terrorist groups. The vulnerability of the nation's milk supply was highlighted in a recent modeling study, where its complex distribution system would magnify the consequences of poisoning by BoNT (Kalb et al., 2005; Wein and Liu, 2005). BoNTs are a serious threat to the US national security due to their potency, remarkable stability, and persistence in the body.

Wein and Liu (2005) modeled a bioterror attack using BoNTs on the nation's milk supply. Modeling of toxins for dispersal into a liquid medium has been previously computed in a terrorist scenario (Dembek et al., 2005) involving a water fountain and contamination at a recreational center (CDC, 1999). Wein and Liu's assessment estimates the amount of toxin required, critically evaluates entry points into the milk supply industry, and details deficiencies in our current detection capabilities required to thwart such an attack (Wein and Liu, 2005).

The most prevalent BoNTs isolated in human botulism are serotypes A, B, and E. The ability of serotypes C and D, in addition to F, to paralyze human skeletal muscle should also be noted (Hilmas, unpublished). Complicating matters is the fact that all BoNTs remain stable in common beverages and retain significant potency for prolonged periods of time (>90 days) at room temperature and in biological fluids (human whole blood and serum) at physiological temperatures (Hilmas et al., 2006b; Williams et al., 2007). In addition, BoNTs possess a remarkable ability to remain within the nerve terminal for extended periods. Keller et al. (1999) showed BoNT protein detectable by western blot for 90 days in rat spinal cord cultures.

Stability of the BoNT protein should be considered in an assessment of the threat posed by intentional release of the toxins. In addition to the remarkable persistence of the toxin in biological fluids and beverages described

previously, BoNT remains a potent environmental threat. BoNT/A was subjected to desiccation to simulate the residue of an intentional release. Following 28 days of drying, the toxin still possessed remarkable paralytic properties (Williams et al., 2007).

The duration of muscle paralysis following intoxication by BoNT/A exceeds that resulting from exposure to other BoNT serotypes (Keller et al., 1999; Fernández-Salas et al., 2004). The remarkable persistence of BoNT/A action has led to its widespread use in the treatment of disorders of muscle tone and movement (Jankovic and Brin, 1997). Although a long duration is desirable in clinical use, the prolonged action of BoNT/A would also make intoxication by this serotype difficult to treat, particularly if it is used as a bioweapon (Franz and Zajtchuk, 1997). The duration of intoxication by BoNT/E is relatively brief (several weeks), whereas BoNT/B is of intermediate duration (Keller et al., 1999). The basis for the differences in serotype persistence is currently unknown. In any case, a bioterrorist attack, involving the most lethal substance known to humankind, would overwhelm the limited resources (i.e., mechanical ventilators) available to treat botulism patients.

29.10 Treatment

There are currently seven known antigenic serotypes of botulinum toxin, designated with the letters A through G, whereby antitoxin to one type does not cross-neutralize any of the others. Only early administration of antitoxin antibody in cases of suspected botulism will minimize the neurologic damage but will not reverse any existing paralysis. Paralysis could persist for weeks to months, and the available treatment consists of supportive care including fluids, TPN, and mechanical ventilation.

29.10.1 Antitoxin

The administration of a heterologous antitoxin was one of the first therapeutic approaches developed for botulism patients and remains the most effective when initiated in the early stages of intoxication. The primary limitation of antitoxin treatment was established in some of the earliest published reports on experimental botulism. One of these reports evaluated the pathogenesis of oral intoxication and the efficacy of antitoxin therapy in monkeys (Dack and Wood, 1928). Antitoxin treatment was not effective when administered after symptoms of botulism were already apparent, despite the fact that circulating toxin could still be detected in many of the animals.

Oberst et al. (1967) investigated the effectiveness of antitoxin therapy, artificial respiration, and supportive treatment in rhesus monkeys after IV type A toxin injection. These treatments were administered to the animals, either alone or in combination, after signs of intoxication were observed. Only one in six monkeys survived after receiving antitoxin injections alone as treatment for overt intoxication with 2.5 LD_{50} of type A toxin (Oberst et al., 1967). A combination of antitoxin therapy and supportive treatment initiated soon after the development of toxic signs protected 8 of 10 animals from death after i.v. injection of 4−5 LD_{50}. Artificial respiration prolonged survival in monkeys with respiratory paralysis but was ineffective as a primary treatment after lethal intoxication; no animals receiving only artificial respiration survived intoxication with 5−24 LD_{50} (Oberst et al., 1967). Untreated animals developed overt signs of intoxication within 20−38 h and died 32−135 h after toxin injection.

While antitoxin treatment was generally ineffective in experimental animals displaying significant clinical signs, several case studies of foodborne botulism indicated that antitoxin therapy remained potentially beneficial in humans even after the onset of illness. Iida et al. (1970) reviewed the high efficacy of antitoxin therapy in type E botulism outbreaks associated with contaminated fish consumption in Japan. A mortality rate of only 3.5% was observed among 85 antitoxin-treated patients in 9 recent foodborne botulism outbreaks, while a rate of 28.9% was reported among 135 untreated patients in 19 previous outbreaks. Iida et al. (1970) noted that all moderately and seriously ill patients in a 1962 foodborne type E botulism outbreak survived after antitoxin treatment. Hatheway et al. (1984) reported on the effectiveness of trivalent (ABE) antitoxin therapy during a 1978 outbreak of type A botulism. Four of seven patients with confirmed disease from type A toxin ingestion were treated with two to four vials of trivalent antitoxin (Hatheway et al., 1984). All four treated patients survived, although one of these individuals continued to suffer from severe paralysis and required ventilatory assistance for several months.

The current CDC therapy for the public is an FDA-approved, bivalent, botulinum equine antitoxin against serotypes A and B. The trivalent antitoxin against types A, B, and E is no longer available. In cases of exposure to any of the other botulinum toxin serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) equine antitoxin, but the time required for typing a toxin subtype would limit its effectiveness in certain cases, such as an outbreak. A parenteral vaccine against the toxin is currently available, but the need exists for newer, nonparenteral vaccines that could be administered orally or via inhalation.

29.10.2 Treatment for infant botulism

Administration of equine antitoxin is not recommended for preexposure prophylaxis. The heterologous serum of antitoxin therapy can lead to a high frequency of adverse

reactions. The equine antitoxin available for use in humans has been reported to cause adverse reactions, such as anaphylaxis, in over 20% of treated patients (Lewis et al., 1980). This problem has been circumvented in the development of a safer approach to the treatment of infant botulism using plasma isolated from human subjects repeatedly immunized with pentavalent toxoid. Equine antitoxin is not used as a treatment for infant botulism due to the high risk of serious adverse reactions and the possibility of long-term sensitization to horse serum-based therapeutics (Arnon et al., 1998). An antiserum product termed BabyBIG (botulism immune globulin), derived from human volunteers immunized with pentavalent toxoid, is available for infant botulism patients. Intravenous BabyBIG therapy has proven extremely effective in counteracting the toxic effects of *C. botulinum* colonization in infants and in avoiding the risk of adverse reactions to equine antitoxin. It is also most effective when administered within 24 h of a high-dose aerosol exposure to the toxin (Gelzleichter et al., 1998a,b).

29.10.3 Vaccines

There are as yet no FDA-approved vaccines to prevent botulism. An investigational pentavalent botulinum toxoid (PBT) product, developed at Fort Detrick, Maryland, is available for persons at risk for botulism (i.e., laboratory workers and soldiers). While determined to be safe and immunogenic, PBT is not useful, nor is it recommended, for postexposure prophylaxis. Antitoxin titers do not develop until a month after the third dose in the vaccine schedule. PBT is reserved for employees at high risk for BoNT exposure, not for the general population. Several factors limit the usefulness of PBT as a vaccine for inoculating the general population. These include a declining potency and immunogenicity in recent years, the need to take multiple doses to maintain titers, and the limited supply of the vaccine.

29.11 Concluding remarks and future directions

The toxicity of botulinum toxins leading to paralysis is due to their ability to block ACh release from peripheral cholinergic nerve endings (Simpson, 2004). Once ingested or inhaled, the toxin binds to epithelial cells, transports to target tissues via the circulatory system, targets the NMJ, and penetrates cellular and intracellular membranes. BoNTs bind to the lipid bilayer of the neuronal cell surface, gain access by receptor-mediated endocytosis, and cleave polypeptides involved in exocytosis of ACh. As a result, botulism leads to a descending flaccid paralysis, starting usually in the bulbar musculature to involve deficits in sight, speech, and swallowing. Paralysis eventually progresses beyond CN palsies to include generalized muscle weakness and loss of critical accessory muscles of respiration. If untreated, death is inevitable from airway obstruction secondary to paralysis of pharyngeal, diaphragm, and accessory respiratory muscles, as well as loss of the protective gag reflex.

The CDC-recommended therapy for the public is a trivalent equine antitoxin against types A, B, and E. In cases of exposure to other BoNT serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) antitoxin. However, the antitoxins are in limited supply and would need to be retrieved from stockpiles. Therefore, the development of safe and effective postexposure therapeutic compounds for BoNT intoxication is of paramount importance to serve the requirements of the military and civilian populations. In conjunction with drug-discovery efforts, there is a parallel exigency to develop appropriate animal models to test the usefulness of various strategies for protection against BoNT intoxication.

29.11.1 Development of animal model test systems

29.11.1.1 Inadequacies of current animal model test systems

Currently, a large number of animal models (mice, rats, guinea pigs, rabbits, and nonhuman primates) have been used for BoNT research, and it is not clear which species is the most appropriate. This is especially problematic since there are marked species differences in the relative potencies of the different serotypes and in their latency of action (the effect of BoNT/B in mice, rabbits, or guinea pigs vs rats; Erdal et al., 1995; Hilmas et al., 2006a). Mice, in particular, are desirable in BoNT research because they offer the most favorable balance between the scientific needs of the experiment and consistency with the existing literature. A variety of mouse strains and sexes have been used for other BoNT studies. The mouse LD_{50} is still used to quantitate the purity of BoNT batches and is the basis of the international standard used in serum neutralization assays of BoNT antitoxin. The mouse phrenic nerve–hemidiaphragm assay has been used to measure the effect of BoNTs on skeletal muscle contraction, and the doses necessary for inhibition are well characterized. The mouse has further advantages over other rodent species like rats.

Rats are not a valuable test system for BoNTs, as they are widely recognized as being insensitive to serotype B (Verderio et al., 2006). On the other hand, skeletal muscles of CD-1 mice, Hartley guinea pigs, and New Zealand White rabbits have similarities to humans, in that their muscles are sensitive to serotypes A, B, C, D, and E (Hilmas et al., 2006b). In vivo and in vitro physiological

assessments of BoNT action in rat have also proved to show inconsistent and erroneous results. In vivo experiments using the rat extensor digitorum longus (EDL) muscle assay showed sensitivities of rat muscle to the B serotype at low doses (10 MU, corresponding to approximately 1−10 pM) (Adler et al., 1996), despite the wide body of literature on the rat to the contrary. In addition, ex vivo rat phrenic nerve−hemidiaphragm preparations are insensitive to BoNT/B, even at very high concentrations in the nanomolar range (Williams et al., 2007).

Another physiological model to evaluate therapeutic candidates against BoNT intoxication is the rat toe spread assay. The rat toe spread assay is problematic as a model test system, however. First, it will not allow for the evaluation of therapeutic candidates against the B serotype since rats are insensitive to BoNT/B. Second, the rat toe spread assay does not involve focal application of BoNT; neighboring muscles are paralyzed due to local diffusion of toxin from the site of i.m. injection. Toe spread in the rat is mediated predominantly by digiti minimi abductor muscles and to a lesser extent by the EDL, the actual muscle injected in the assay. Intramuscular injection of rat EDLs with BoNT will primarily paralyze the EDL and, to a lesser extent, the digiti mini muscles, the true abductors of toe spread, by local diffusion. Therefore, EDL muscles injected with BoNTs would tend to show an erroneously early recovery of toe spread as the primary effectors of toe spread recover sooner compared to injected EDL muscles. To date, there is no acceptable in vivo model to test the efficacy of inhibitory compounds.

29.11.1.2 Advantages of the mouse hemidiaphragm assay

Current approaches to the inhibition of BoNT activity involve a number of strategies, each with potential advantages and disadvantages. Ultimately, model test systems that can incorporate each of these potential approaches are needed to evaluate the relative merit of potential therapeutic compounds. Since the presynaptic terminal is the primary target for BoNTs, a test system based on toxin action at presynaptic terminals is indicated. Such systems should permit testing of all relevant aspects of toxin (internalization, activity, and overcoming the inhibition of transmitter release), should be simple and reliable, and should permit rapid evaluation of novel therapeutics or their precursor compounds.

Due to the high sensitivity of mammalian synapses to the actions of BoNTs, due in part to the presence of high-affinity binding sites for toxin on the cell surface and to the intracellular presence of the appropriate enzymatic substrates, the test model systems should be of mammalian origin. Muscle is the ideal test system for BoNT since it is the most sensitive in vivo target for neurotoxin action. In addition, inhibition of the diaphragm muscle is the proximal cause of death in botulism (Simpson, 1986). Furthermore, a positive result with BoNT on muscle implies that the toxin is correctly folded and the binding, catalytic, and translocation domains are all intact. Enzyme-linked immunosorbent assays (ELISAs), on the other hand, detect only components of the toxin and may provide positive results when the toxin has in fact lost its ability to intoxicate (Kalb et al., 2005). The mouse phrenic nerve−hemidiaphragm assay is a favorable model test system to evaluate therapeutics against BoNT-induced paralysis.

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Chapter 30

Onchidal and fasciculins

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30.1 Introduction

Onchidal and fasciculins are natural toxins that produce their toxicity in mammalian systems primarily by virtue of acetylcholinesterase (AChE) inhibition. AChE hydrolyzes acetylcholine (ACh), thereby regulating the concentration of the transmitter at the synapse. Termination of activation normally depends on dissociation of ACh from the receptor and its subsequent diffusion and hydrolysis, except in diseases where ACh levels are limited or under AChE inhibition, conditions that increase the duration of receptor activation (Silver, 1963).

The toxins and toxicants that inhibit AChE are called *anticholinesterase (anti-ChE)* agents. They cause accumulation of ACh in the vicinity of cholinergic nerve terminals, and thus, are potentially capable of producing effects equivalent to excessive stimulation of cholinergic receptors throughout the central nervous system (CNS) and peripheral nervous system (Long, 1963). Nevertheless, several members of this class of compounds are widely used as therapeutic agents; others that cross the blood–brain barrier (BBB) have been approved or are in clinical trial for the treatment of Alzheimer's disease (AD). The treatment approaches in the neurodegenerative pathology of AD continue to be primarily symptomatic, with the therapeutic strategies based on the cholinergic hypothesis, and specifically on AChE inhibition (Lane et al., 2004).

AChE can be classified in several ways. Mechanistically, it is a serine hydrolase. Its catalytic site contains a catalytic triad—serine, histidine, and an acidic residue—as do the catalytic sites of the serine proteases (such as trypsin), several blood clotting factors, and others. However, the acidic group in AChE is a glutamate, whereas in most other cases, it is an aspartate residue. The nucleophilic nature of the carboxylate is transferred through the imidazole ring of histidine to the hydroxyl group of serine, allowing it to displace the choline moiety from the substrate, forming an acetyl-enzyme intermediate. A subsequent hydrolysis step frees the acetate group. Understanding of the catalytic properties of the protein has assisted in our understanding of its inhibition by organophosphate (OP) and carbamate (CM) inhibitors. However, several questions remain to be answered regarding AChE catalysis, such as the mechanism behind the extremely fast turnover rate of the enzyme (Fair et al., 1994). Despite the fact that the substrate has to navigate a relatively long distance to reach the active site, AChE is one of the fastest-reacting enzymes (Nair et al., 1994). One theory to explain this phenomenon has to do with the unusually strong electric field of AChE. It has been argued that this field assists catalysis by attracting the cationic substrate and expelling the anionic acetate product (Ripoll et al., 1993). Site-directed mutagenesis, however, has indicated that reducing the electric field has no effect on catalysis. However, the same approach has indicated an effect on the rate of association of fasciculin, a peptide that can inhibit AChE and produce muscle fasciculations (Shafferman et al., 1994).

Naturally occurring irreversible inhibitors of AChE are toxins that are often selective inhibitors of protein function, and this property can often be exploited for a variety of purposes (Pita et al., 2003). For instance, physostigmine (also called eserine), is a naturally occurring alkaloid reversible inhibitor of AChE that has been used in understanding the kinetic mechanism of AChE. Through its action at both the central and peripheral cholinergic receptors, physostigmine reverses anticholinergic activity and ameliorates the coma, delirium, and seizures that accompany severe toxicity. Antidotes for such poisoning have been developed to accelerate enzyme regeneration. The slow reactivation of AChE is the basis of the use of physostigmine and pyridostigmine (a physostigmine derivative) as a prophylactic in anticipation of the

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use of these toxins. It is argued that the psychological stress associated with warfare impaired the integrity of the BBB, allowing the peripheral-acting inhibitor of AChE to penetrate the brain, where it activated AChE transcription (Kaufer et al., 1998). For further details on toxins/toxicants and the BBB, see Chapter 48, Blood–brain barrier damage and dysfunction by chemical toxicity.

30.2 Background

This chapter covers natural agents that prolong the existence of ACh after it is released from cholinergic nerve terminals. Natural toxins are chemical agents of biological origin (including chemical agents and proteins), and can be produced by all types of organisms; such is the case for onchidal and fasciculins. Although a chemical and a protein, respectively, onchidal and fasciculins share the same toxic effect of inhibiting AChE, which is concentrated in synaptic regions and is responsible for the rapid catalysis of the hydrolysis of ACh. As such, the natural toxins onchidal and fasciculins can produce disorders of neuromuscular transmission that are clinically categorized as either presynaptic or postsynaptic; some toxins simultaneously affect both sites. Chemical agents associated with neuromuscular transmission syndromes include the fasciculins, crotoxin, taipoxin, tubocurarine, and OP compounds (Anadón and Martínez-Larrañaga, 1985).

These aforementioned natural anti-ChE agents can be developed for a different purpose, including extensive application as toxic agents (i.e., potential chemical weapons). One of the first interesting structures with cholinergic properties isolated from a marine source is onchidal. This compound was first isolated from the mollusk *Onchidella binneyi* and has an acetate ester similar to ACh. Upon isolation, onchidal was discovered as being an active, site-directed, and irreversible inhibitor of AChE (Abramson et al., 1989).

Neurotoxins from snake venoms have proved to be valuable tools for the understanding of synaptic transmission mechanisms. Likewise, the powerful inhibitory action of fasciculins against mammalian AChE makes them potentially useful for pharmacological and neurochemical research. Studies of their biochemical and electrophysiological effects on the CNS and biochemical characterization are now being carried out.

Natural toxins can be extremely potent, and many of them are effective at far lower dosages than conventional chemical agents. Natural toxins, as compounds of biological origin, are often classed as biological agents, but they are not infectious and are more similar to chemicals with respect to their military potential for tactical use; therefore, they should be considered to be chemical agents that could be used for warfare. The Chemical Weapons Convention (CWC 2003) (available at www.fas.harvard.edu/~hsp/cwc/cwcbyart.html) also includes natural toxins as chemical agents, specifically including the onchidal and fasciculin toxins in its control regime, along with other highly toxic chemicals.

30.2.1 Onchidal

Onchidal is a toxic component of a poisonous marine opisthobranch mollusk. Like other opisthobranchs, the *Onchidiacea* family of mollusks does not have the protection of a hard external shell as most mollusks do. They rely instead on the production of a defensive secretion. When the animal is disturbed, it emits a viscous fluid from specialized glands. In two species of *Onchidella* (*Onchidella floridanun* and *Onchidella borealis*), this defensive secretion has been shown to act as a deterrent to potential predators, including fish and crabs. Chemically, it is a simple lipophilic acetate ester (Fig. 30.1).

Onchidal has been identified as the major lipid-soluble component of the defensive secretion of mollusk *O. binneyi*, and it has been proposed as the compound responsible for the chemical protection of *Onchidella* species. *O. binneyi* is an opisthobranch mollusk that inhabits the rocky intertidal zone near the area of Baja California, Mexico. The defensive secretion was obtained in the field by squeezing the mollusk and collecting the mucus discharge in capillary tubes, exhibiting biological properties including feeding deterrence, and antibacterial and anticholinesterase activities. Large quantities of this material could be obtained after the extraction of intact animals with acetone (Ireland and Faulkner, 1978). However, the distribution of onchidal in different species of *Onchidella* was not reported and, apart from inhibiting the growth of *Staphylococcus aureus* (the IC_{50}value was between 0.21 and 0.63 μg/mL), which implies that onchidal is a potent inhibitor of Gram-positive microorganisms, no biological activity of onchidal was described. Additional studies demonstrated that onchidal is contained in several different species of *Onchidella* and that, once purified, it is toxic to fish (Abramson et al., 1989). Onchidal can be found in four of the eight known species of *Onchidella*

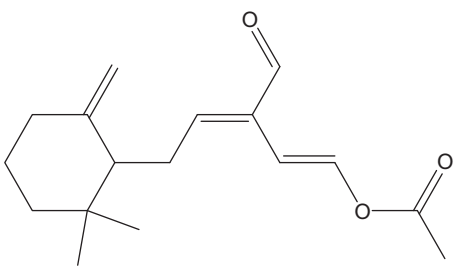

FIGURE 30.1 The chemical structure of onchidal.

collected from different countries (Table 30.1). In addition, the Abramson study found onchidal to be toxic to goldfish. Although goldfish are not potential predators of *Onchidella*, these results demonstrate that onchidal has a distribution and a biological activity consistent with its proposed role in the chemical defense of *Onchidella*.

30.2.2 Fasciculin

The venom of the eastern green mamba snake (*Dendroaspis angusticeps*, *Dendroaspis polylepis*, *Dendroaspis viridis*, *Dendroaspis jamesoni*), with a habitat almost exclusively arboreal, belongs to the three finger toxin family (3FTx), and contains a mixture of neurotoxic compounds, including postsynaptic cholinoreceptor α-neurotoxins, dendrotoxins, fasciculins, and muscarinic toxins (Hawgood and Bon, 1991). Forty-two different proteins were identified in the venom of *D. angusticeps*, in addition to the nucleoside adenosine. The most abundant proteins belong to the 3FTx (69.2%) and the Kunitz-type proteinase inhibitor (16.3%) families. Several subsubfamilies of the 3FTxs were identified, such as Orphan Group XI (Toxin F-VIII), AChE inhibitors (fasciculins), and aminergic toxins (muscarinic toxins, synergistic-like toxins, and adrenergic toxins) (Lauridsen et al., 2016). No α-neurotoxins were identified. Proteins of the Kunitz-type proteinase inhibitor family include dendrotoxins (Lauridsen et al., 2016). Effects at the NMJ include AChE inhibition by fasciculins and increased presynaptic release of ACh by dendrotoxins (polypeptides that facilitate ACh release in response to nerve stimulation; inhibit voltage-gated KV11, KV12, and KV16 potassium channels and block muscarinic receptors); together with the high ACh content of mamba toxin (6−24 mg/g), these effects are synergistic and enhance neurotoxicity and lethality. Moreover, the venom may contain other components that have a synergistic action with dendrotoxin.

Toxins that facilitate neuromuscular transmission are a characteristic component of mamba venom. The four known fasciculins bind to a peripheral regulatory anionic site of AChE in a noncompetitive and irreversible manner (Hawgood and Bon, 1991). The dendrotoxins comprise the second group of facilitatory neurotoxins and are present in most mamba venom (with the exception of *D. jamesoni*); they inhibit voltage-dependent K^+ channels in motor nerve terminals and facilitate ACh release at the NMJ. Postsynaptic toxins present in mamba venom bind to and block nicotinic acetylcholine receptors (nAChRs). The muscarinic toxins present in mamba venom are small proteins (7 kDa) that selectively bind to muscarinic cholinergic receptors and may constitute up to 1% of the venom protein (Adem and Karlsson, 1985; Jerusalinsky and Harvey, 1994). About 12 muscarinic toxins have been isolated. M1 toxin binds noncompetitively and with high affinity to the M_1 muscarinic receptor subtype. MTx1 and MTx2 show high affinity for both muscarinic M_1 and M_3 receptors; little is known about the receptor selectivity of MTx3 and MTx4. Dpα and Dpβ are also muscarinic agonists, displaying similar affinity for both the M_1 and M_2 receptor subtypes. The last two agonists, DpMTx and DvMTX, are selective muscarinic agonists present in the venom of some mamba species; these agonists also show affinity for the M_1 muscarinic receptor subtype.

The fasciculins are a family of closely related ~6750-Da peptides isolated from the venom of mambas (genus *Dendroaspis*), and are named after the long-lasting muscle fasciculations they produce in mice (Rodriguez-Ithurralde et al., 1983). They are potent and selective inhibitors of AChE. Fasciculins are 61-residue-long polypeptides. They share a three-looped structural motif with other toxins, such as α-neurotoxins, cytotoxins, and muscarinic toxins, directed to diverse specific targets. Four fasciculins are known (Table 30.2), which differ only by one to three residues and show selective and potent anti-AChE activity: Fas1 and Fas2 from the venom of *D. angusticeps* (the Eastern green mamba) contains 61 amino acid residues, including eight half-cystines (Rodriguez-Ithurralde et al., 1983), ToxC from the venom of *D. polylepis polylepis* (the black mamba, with a primarily terrestrial habitat) (Joubert and Taljaard, 1978), and Fas3, which was isolated from a particular batch of *D. viridis* (the Western green mamba) venom and found to have the same primary structure as ToxC (Marchot et al., 1993). No fasciculin has been found in other *D. viridis* venoms (Marchot et al., 1993), or in *D. jamesoni* (Jameson's mamba).

TABLE 30.1 Concentration of onchidal in different species of *Onchidella* (Abramson et al., 1989).

Organism	Collection site	Onchidal concentration (μg/animal)
Onchidella binneyi	Baja California, Mexico	230
Onchidella borealis	Central California, USA	33
Onchidella nigricans	New Zealand	18
Onchidella patelloides	Australia	42

TABLE 30.2 Types of fasciculin identified.

Fasciculins characterized	From the mamba snake venoms (Elapidae family) (*Dendroapsis* genus)
Fas1	*Dendroaspis angusticeps* (Eastern green mamba)
Fas2 (formally F_7 toxin)	*Dendroaspis angusticeps* (green mamba)
Fas3 (formally Toxin C)	*Dendroaspis polylepis* (black mamba)
Fas4	*Dendroaspis viridis* (Western green mamba)

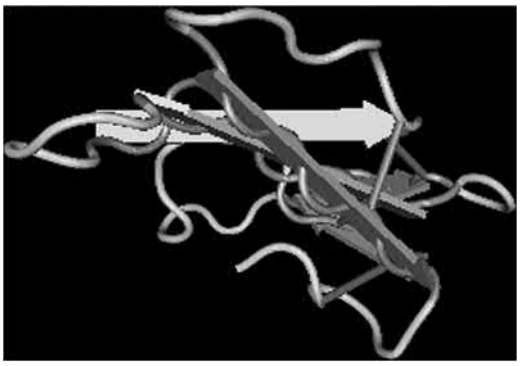

FIGURE 30.2 The 3-D protein structure of Fas1 derived from green mamba (*Dendroaspis angusticeps*) snake venom. *Obtained from the public domain at the U.S. National Library of Medicine, National Center for Biotechnology Information, MMDB.*

Angusticeps-type toxins constitute a group of toxins typical for mamba venoms. They consist of 59−61 amino acid residues and four disulfides, and show sequence homology with short-chain postsynaptic toxins and cardiotoxins, but they are immunochemically distinct from these toxins. Eight toxins of this type have been sequenced and structurally classified into four subgroups (Joubert and Taljaard, 1978). Pharmacological studies on these *angusticeps*-type toxins have revealed that only toxins F_7 and C, which belong to subgroup I, exhibit a potent inhibition of cholinesterase (ChE) of various tissues, except for that of chick skeletal muscle. Both F_7 and C stimulate mouse and toad skeletal muscles by their anti-ChE activity, as well as possibly by facilitating ACh release from nerve terminals (Lee et al., 1985). Fasciculins account for 3%−6% of the venom protein, and the relative proportion between Fas1 and Fas2 is about one-third (Cerveñanský et al., 1994).

The structural similarity between onchidal (an acetate ester) and ACh suggested that the toxicity of onchidal could result from inhibition of either nAChRs or AChE. Although onchidal (1.0 mM) did not prevent the binding of ^{125}I-α-bungarotoxin to nAChRs, it inhibited AChE in a progressive, apparently irreversible manner. The apparent affinity of onchidal for the initial reversible binding to AChE (K_d) was approximately 300 μM, and the apparent rate constant for the subsequent irreversible inhibition of enzyme activity (K_{inact}) was approximately 0.1 min^{-1}.

Fasciculins are a family of closely related peptides that are isolated from the venom of mambas and exert their toxic action by inhibiting AChE. The crystal structure of Fas2 from green mamba (*D. angusticeps*) snake venom was first resolved in 1992 (Le Du et al., 1992). The three-dimensional structure of Fas1, obtained from the US National Library of Medicine, National Center for Biotechnology Information, Molecular Modeling Database 3-D Structure Database (MMDB), is illustrated in Fig. 30.2.

Fasciculins belong to the structural family of three-fingered toxins from Elapidae snake venoms. Fasciculins selectively inhibit mammalian AChE with Ki values in the picomolar range and include the α-neurotoxins that block the nAChR and the cardiotoxins that interact with cell membranes. Elapid venoms contain AChEs, and typically affect peripheral nerves rather than the CNS because neurotoxins do not penetrate the BBB. The features unique to the known primary and tertiary structures of the fasciculin molecule were analyzed by Harald and colleagues (1995). Loop I contains an arginine at position 11, which is found only in the fasciculins and could form a pivotal anchoring point to AChE. Loop II contains five cationic residues near its tip, which are partly charge-compensated by anionic side chains in loop III. In contrast, the other three-fingered toxins show full charge compensation within loop II. The interaction of fasciculin with the recognition site on AChE was investigated by estimating a precollision orientation, followed by determination of the buried surface area of the most probable complexes formed, the electrostatic field contours, and the detailed topography of the interaction surface. This approach has led to testable models for the orientation and site of bound fasciculin.

30.3 Mechanism of action and biological effects

30.3.1 Onchidal

Onchidal belongs to the group of natural nonprotein neurotoxins and is an irreversible inhibitor of the AChE enzyme, with a novel mechanism of action. It has been suggested, however, that its toxicity could be a consequence of the inhibition of either nAChRs or the AChE enzyme. It was found that onchidal acts as an electrophile, reacting rapidly with the model nucleophile

n-pentylamine forming diasteromeric aminated pyrrole adducts. Onchidal and analogs reacted with the model protein lysozyme, forming covalent adducts and leading to protein cross-linking (Cadelis and Copp, 2018). The electrophilic reactivity of onchidal suggests a potential to induce DNA damage, which is discussed in Section 30.5.

Incubation of AChE with onchidal resulted in the production of acetate, demonstrating that onchidal was a substrate for AChE, and approximately 3250 mol of onchidal were hydrolyzed/mol of enzyme irreversibly inhibited. OP and CM inhibitors of AChE have partition ratios (mol of toxin hydrolyzed/mol of enzyme irreversible inhibited) that approach unit. Therefore, the relatively high partition ratio for onchidal suggests that the mechanism of inhibition utilized by onchidal may be distinctly different from other irreversible inhibitors (Walsh, 1984). The rate of hydrolysis of onchidal (K_{cat}) was 325 min^{-1}; this value is relatively slow, suggesting that onchidal is not a very good substrate. The ability of AChE to hydrolyze onchidal raised the question of whether inhibition of enzyme activity resulted from onchidal itself, or from a product of the enzymatic hydrolysis of onchidal. Enzyme kinetics revealed that onchidal was unable to completely inhibit higher concentrations of AChE. From the experiments performed by Abramson et al. (1989), onchidal was in molar excess and completely hydrolyzed. Thus, irreversible inhibition of enzyme activity resulted either from onchidal itself, or from a reactive intermediate produced during the hydrolysis of onchidal (Walsh, 1984).

In another investigation, irreversible inhibition of enzyme activity was prevented by co-incubation with reversible agents that either sterically block (edrophonium and decamethonium) or allosterically modify (propidium) the ACh site (Barnett and Rosenberry, 1977). Enzyme activity was not regenerated by incubation with oxime reactivators; therefore, the mechanism of irreversible inhibition does not appear to involve acylation of the active site serine.

Because onchidal is an acetate ester similar to ACh, and because cholinergic neurotransmission is often the site of action of natural products involved in chemical defense, Abramson et al. (1989) investigated the ability of onchidal to inhibit AChE and the nAChR. Although onchidal did not prevent the binding of ^{125}I-α-bungarotoxin to nAChRs, it was shown to be an active site-directed irreversible inhibitor AChE. The structure and pharmacology of onchidal suggest that inhibition of AChE results from a novel covalent reaction between onchidal and an amino acid within the ACh-binding site. The onchidal could potentially be exploited in the design of a new class of natural anti-ChE agents and in the identification of amino acids that contribute to the binding and hydrolysis of ACh.

30.3.2 Fasciculin

Various toxins in snake venoms exhibit a high degree of specificity in the cholinergic nervous system. The α-neurotoxins from the *Elapidae* family interact with the agonist-binding site on the nicotinic receptor. α-Bungarotoxin is selective for the muscle receptor and interacts with only certain neuronal receptors, such as those containing α7 through α9 subunits. Neuronal bungarotoxin shows a wider range of inhibition of neuronal receptors. A second group of toxins, the fasciculins, inhibits AChE. A third group of toxins, termed the *muscarinic toxins* (MT_1−MT_4), are partial agonists and antagonists for the muscarinic receptor. Venoms from the *Viperaridae* family of snakes and the fish-hunting cone snails also have relatively selective toxins for nicotinic receptors.

Other reversible inhibitors, such as propidium and fasciculin, bind to the peripheral anionic site on AChE. This site resides at the lip of the gorge and is defined by tryptophan 286 and tyrosines 72 and 124 (Taylor, 2001).

A large number of organic compounds reversibly or irreversibly inhibits AChE (Long, 1963), which binds either to the esteratic or the anionic subsite of the AChE catalytic site or to the peripheral site of the enzymes. Most are synthetic substances, sometimes with insecticidal properties. Few natural inhibitors of AChE are known and, to date, fasciculins are the only known proteinic AChE inhibitors. They have been shown to display a powerful inhibitory activity toward mammalian AChE. Iodination of Fas3 provided a fully active and specific probe of fasciculin-binding sites on rat brain AChE (Marchot et al., 1993). These authors demonstrate that fasciculins bind on a peripheral site of AChE, distinct from the catalytic site and, at least partly, common with the sites on which some cationic inhibitors and the substrate in excess bind; since phosphorylation of the catalytic serine (esteratic subsite) by [1,3-^{3}H]diisopropyl fluorophosphate can still occur on the Fas3. In the AChE complex, the structural modification induced by fasciculins may affect the anionic subsite of the AChE catalytic site.

Cholinesterases have a very different sensitivity toward fasciculins. AChEs from rat brain, human erythrocytes, and electroplax of electric eel (*Electrophorus electricus*) are inhibited by fasciculins with a Ki of about 10^{-11} M and pseudocholinesterases as human serum ChE are inhibited by fasciculins with a Ki of about 0.5 μM. A second group of enzymes is partially (10%−30%) inhibited by low concentrations (< 0.5 nM) of fasciculin. Increasing the concentration of fasciculins to a toxic level of about 1 nM inactivates the enzymes to 90%−110% of their initial activity. AChEs from guinea pig ileum, ventricle, and uterus behave similarly. A third group consists of enzymes insensitive to fasciculin; AChEs from chick

biventer cervicis muscle and brain and from insects, heads of *Musca domestica* (common housefly) and cobra (*Naja naja*) venom. The biochemical mechanism of fasciculins involves displacement of propidium from its binding site on AChE. Since propidium is a probe from a peripheral anionic site, it is concluded that fasciculins also bind to the same site. The different sensitivities of ChE to fasciculin should depend on the nature of their peripheral sites. Fasciculins are basic proteins of 61 amino acid residues and 4 disulfides, highly homologous to short α-neurotoxins and cardiotoxins. Indeed, a large number of AChE inhibitors are cations (e.g., neostigmine, physostigmine, and propidium).

The binding between fasciculin and AChE is strong, as indicated by a Ki of about 10^{-11} M. This should result from interaction of several amino acid residues in the toxin with the enzyme. A modification of one of these residues should not abolish the activity, but it should significantly decrease it. However, the decrease in activity can also depend on structural perturbations caused by the modification. Chemical modification and structural data suggest that Lys32 and Lys51 have a functional role (Cerveňanský et al., 1994). This author acetylated the amino groups of Fas2 with acetic anhydride. The monoacetyl derivatives of the ε-amino acids (Lys25, Lys32, Lys51, and Lys58) retained between 28% and 43% of the initial activity, and that of the α-amino group retained 72%. Acetylation of Lys25 that has the most reactive amino group decreased the activity by 65%, apparently without producing structural perturbation since the circular dichroism spectrum was not affected. The three-dimensional structure shows a cationic cluster formed by Lys32, Lys51, Arg24, and Arg28. A comparison of 175 sequences of homologous toxins shows that Lys32 is unique for fasciculin. Acetylation of lysine residues in the cluster had a large effect and reduced the activity by 72% (Lys32) and 57% (Lys51).

Fasciculins inhibit AChE from mammals, electric fish, and some snake venoms with Ki values in the picomolar to nanomolar range; in contrast, the AChEs of avian, insect, and some other snake venoms are relatively resistant, and high micromolar concentrations are required to inhibit mammalian butyrylcholinesterases (BuChE) (Marchot et al., 1993). Dissociation constants of Fas1 and Fas3 are twofold and 60-fold lower, respectively, than that of Fas2 for synaptosomal rat brain.

An examination of fasciculin association with several mutant forms of recombinant deoxyribonucleic acid (DNA)-derivated AChE from mice shows that it interacts with a cluster of residues near the rim of the gorge on the enzymes; the aromatic residues, Trp286, Tyr72, and Tyr124, have the most marked influence on fasciculin binding, whereas Asp74, a charged residue in the vicinity of the binding site that affects the binding of low-molecular-weight inhibitors, has little influence on fasciculin binding. The three aromatic residues are unique to the susceptible AChE and, along with Asp74, constitute part of the peripheral anionic site. Fasciculin falls into the family of three-loop toxins that include the receptor-blocking α-toxins and cardiotoxins. A binding site has evolved on fasciculin to be highly specific for the peripheral site on AChE. Acetylthiocholine affects rates of fasciculin binding at concentrations that cause substrate inhibition. In the case of the mutant ChE, where rates of fasciculin dissociation are more rapid, steady-state kinetic parameters also show ACh−fasciculin competition to be consistent with occupation at a peripheral or substrate inhibition site rather than the active center (Radic et al., 1994).

Fasciculin-induced inhibition of AChE is prevented by chemical modification of the enzyme at a peripheral site (Durán et al., 1994). The specific interaction of Fas2 with peripheral sites present in *E. electricus* AChE (Ki; 0.04 nM fasciculin) was investigated by chemical modification with *N*,*N*-dimethyl-2-phenylaziridium in the presence of active or peripheral anionic site protective agents. An enzyme was obtained that was compared to native AChE and was 10^6 times less sensitive to Fas2. This enzyme was fully inhibited by edrophonium and tacrine and was 25−170 times less sensitive to several peripheral site ligands. It seems Fas2 binding to an AChE peripheral site partially overlaps the site of other peripheral site ligands, including ACh.

30.4 Experimental and human toxicity

30.4.1 Experimental

Administration of Fas1 and Fas2 to mice at doses of 1−3 mg/kg and 0.05−2.0 mg/kg, respectively, after intraperitoneal (i.p.) injection caused severe, generalized, and long-lasting fasciculations (5−7 h), followed by gradual recovery to normal behavior. In vitro preincubation with fasciculins at concentrations of 0.01 μg/mL inhibited brain and muscle AChE up to 80%. Histochemical assay for AChE showed an almost complete disappearance of the black-brown precipitate at the neuromuscular endplate after in vitro incubation with fasciculins. Fasciculins represent a new type of AChE inhibitor exerting muscle fasciculations through a powerful inhibition of enzyme activity at the neuromuscular endplate, interfering with the normal hydrolysis of ACh molecules. Fasciculins have also been demonstrated to be powerful inhibitors of brain AChE (Rodriguez-Ithurralde et al., 1983).

The cause of death due to toxin F_7, an *angusticeps*-type toxin isolated from the venom of *D. angusticeps*, was studied in anesthetized mice (Lee et al., 1986). The carotid arterial blood pressure, electrocardiography, and the respiratory movements were recorded. Within a few

minutes after intravenous (i.v.) injection of F_7 (1 mg/kg), both the rate and amplitude of the respiratory movements decreased, and respiratory arrest took place within 15 min in most cases. Before respiratory arrest, marked bradycardia with various types of arrhythmia and oscillation of blood pressure were observed. Artificial ventilation could abolish these cardiovascular changes and maintain the blood pressure for a long period. Toxin F_7 caused a transient and slight increase in arterial blood pressure, which could be prevented by hexamethonium. Intracisternal application of toxin F_7 (1 mg/kg) caused long-lasting hypertension and bradycardia and the respiratory arrest time was significantly longer than after i.v. injection. A large dose (50 mg/kg, i.p.) of atropine, but not smaller doses (5−10 mg/kg), protected mice against respiratory failure induced by toxin F_7.

In rats, the phrenic nerve discharge was prolonged during respiratory depression. Since the toxin F_7 has a potent anti-ChE activity, it is concluded that the respiratory failure induced by toxin F_7 is peripheral in origin; this was chiefly, if not entirely, due to its anti-ChE activity. Despite its lack of α-neurotoxins, the venom of *D. angusticeps* is quite effective in killing mice rapidly after injection, as observed in acute toxicity experiments with crude venom, where the controls receiving 4 LD_{50s} of venom on average died within 10 min. Previous studies have highlighted two main toxic activities when *D. angusticeps* whole venom is tested in experimental systems. On various nerve-muscle preparations, this venom augmented the responses to indirect stimulation (Barrett and Harvey, 1979), possibly due to the combined action of dendrotoxins and fasciculins. Thus, the combined action of the various neurotoxin types present in *D. angusticeps* venom may result in a complex series of neuromuscular and cardiovascular effects. The combined action of these toxins results in effective prey immobilization despite the absence of the action of α-neurotoxins. The observation of this mixture's toxicity, that likely involves synergistic effects among the toxins, complicates the selection of the most relevant toxins toward which antibodies should be raised in order to abrogate venom toxicity (Lauridsen et al., 2016).

Strydom (1976) performed the purification of *D. polylepis polylepis* venom and found 12 low-molecular-weight proteins, of which 11 have subcutaneous LD_{50} values of less than 40 μg/g in the mouse. Clinically, mamba bites may not provoke a major local reaction. If neurotoxins are injected by the bite, clinical symptoms appear within minutes to hours.

30.4.2 Human

Envenomation by mambas cause rapid onset of perioral and generalized paresthesias, nausea, vomiting, hypersalivation, and hyperacusis followed by generalized weakness, ptosis, dysphagia, diaphoresis, goose flesh, and drowsiness. Despite a low number of human envenomations reported, *D. angusticeps* is classified as a "category 1" snake, which is the highest level of medically important snakebites, according to the WHO (WHO, 2016). Elapids are present in restricted areas of Africa. In particular they are found with high abundance in Kenya, Tanzania, Mozambique, Malawi, Eastern Zimbabwe, and the Republic of South Africa. Consequently, this snake species is of high epidemiological relevance to the region (WHO, 2016). Clinical features of severe envenomation by *D. angusticeps* can lead to rapid mortality within only 30 min of a bite (Spawls and Branch, 1995). The typical clinical manifestations include swelling of the bitten area, dizziness, nausea, difficult breathing, irregular heartbeat, and respiratory paralysis (Spawls and Branch, 1995). These life-threatening symptoms may escalate rapidly, but deaths are rare when effective antivenom is administered in a timely fashion. Clinical signs of impairment of neuromuscular transmission (ptosis, ophthalmoplegia, bulbar symptoms, or generalized weakness) dictate administration of antivenom (Ludolfph, 2000). The venom of *Elapidae* (coral snakes), is known to be a potential neurotoxin and may cause paresthesias, weakness, cranial nerve dysfunction, confusion, fasciculations, and lethargy. Often mild local findings, diplopia, ptosis, and dysarthria are common early symptoms. Patients die because of respiratory paralysis. In these cases, early and aggressive airway management is vital. Symptoms may be delayed by 8−12 h (Cameron, 2006).

Given the medical importance of *D. angusticeps*, it is necessary to have a thorough understanding of the composition of its venom, as well as of the underlying mechanisms for venom pathophysiology in human victims. Furthermore, preclinical assessment of antivenoms is critical for predicting the efficacy of snakebite envenoming therapy, which may be used to guide clinicians in the treatment of snakebites by *D. angusticeps*. Currently, only the SAIMR Polyvalent Snake Antivenom from the South African Vaccine producers is claimed to be effective against *D. angusticeps*. However, it is possible that other polyvalent antivenoms raised against the venoms of other mamba species may be effective in neutralization of *D. angusticeps* venom. The venom of *D. angusticeps* has not undergone a full proteomics evaluation, and its quantitative protein composition is not known. Nevertheless, several biochemical and pharmacological studies have been performed on different toxins from *D. angusticeps* venom (Harvey, 1990). These studies report that this venom contains several neurotoxins, such as the fasciculins and dendrotoxins (Harvey, 1990; Harvey and Anderson, 1991; Cerveñansky et al, 1991), which are unique to the *Dendroaspis* genus. This venom also

contains a number of other toxins of the three-finger toxin family (3FTx), such as muscarinic toxins, adrenergic toxins, and synergistic-type toxins (Blanchet et al, 2014).

30.5 Computational toxicology assessment

Toxicity databases and computer-assisted (in silico) computational predictive toxicology modeling are now becoming increasingly important to risk assessors when risk assessment of a chemical using experimental animal toxicological studies is unclear because the data are equivocal or even absent (Valerio, 2009; Bailey et al., 2005; Arvidson et al., 2008). There are many reasons for the development of in silico toxicology systems. Over 10 years ago a law passed by the European Union called Registration, Evaluation, Authorization, and restriction of Chemicals (REACH), entered into force on June 1, 2007. The goal of this regulation was to identify and more effectively avoid the risks that the toxic properties of chemicals pose to humans and the environment (Lahl and Gundert-Remy, 2008). Under REACH, firms that manufacture or import more than 1 ton of a chemical substance per year in the European Union are required to assess the chemical's potential toxicological or environmental adverse effects, and to register this information in a central database (Saiakhov and Klopman, 2008). REACH has had an impact on the widespread use of computational predictive modeling because it rules that no animal test should be used if it can be replaced with other techniques, such as reliable computer-based predictions (Lahl and Gundert-Remy, 2008; Saiakhov and Klopman, 2008). Therefore, the use of computer-assisted computational predictive toxicology software has attracted considerable attention worldwide because when applied under the appropriate circumstances, it is consistent with the 3Rs (reduce, refine, replace) concept for animal testing.

Since the acute and chronic toxic potentials of onchidal in experimental animal studies are rather limited, strategies using in silico assessment of its toxic potential could be envisioned. Computational-based strategies with pharmaceuticals and natural product toxins have already been developed for use in safety evaluation and risk assessment (Valerio, 2009, 2013; Valerio et al., 2007, 2013; Arvidson et al., 2010; Demchuk et al., 2008; Bailey et al., 2005; Valerio and Cross, 2012; Choi et al, 2013; Valencia et al, 2013). These strategies can be considered for an in silico risk assessment of onchidal. The electrophilic properties reported for onchidal (Cadelis and Copp, 2018), and as illustrated on its molecular structure (Fig. 30.1), raises the question of whether the structural features are associated with genotoxicity or carcinogenicity. Computational toxicology predictive systems were developed as an extension of Ashby-Tennant alerts that were based on compounds many of which bear electrophilic moieties. Therefore, the potential of onchidal to act as a mutagenic agent through direct DNA reactivity could be assessed using a computational model for DNA reactivity (i.e., an Ames test). One approach is to combine different predictive models for the same toxicological endpoint to determine if a consensus prediction regarding the mutagenic or other toxicological effects measured by a computer model is possible.

There are computational software that employ human rule-based approaches with software that utilize statistical algorithm-based predictions using quantitative structure−activity relationship (QSAR) models for the toxic potential of the compound of interest. There are computational models which are not based on a statistical association and these are referred to as structure−activity relationship (SAR) human expert systems, as predictions arise from the coding of a set of human expert knowledge into rules (i.e., structural alerts). If the outcome from a QSAR and SAR structural assessment with expert interpretation is negative, this can indicate that the assessment is sufficient to conclude there is no concern and to treat the compound as negative for the toxicological endpoint. If the outcome from the complementary in silico QSAR and SAR assessments is positive, then the compound could raise a concern to an assessor and potentially be prioritized for further standardized genetic toxicology testing or simply inform the risk assessment procedure.

The use of in silico toxicology methods has taken a real practical role and utility as a tool in facilitating decisions in chemical risk assessment. There are a variety of computational toxicology predictive software and data-mining databases available at either no cost or commercially for a fee (Choi et al., 2013; Valencia et al., 2013; Richard et al., 2008; Marchant et al., 2008; Saiakhov and Klopman, 2008; Shi et al., 2008; Mostrag-Szlichtyng et al., 2010). However, the information obtained from a predictive and data-mining in silico assessment must be used judiciously in order to serve its true purpose, which is to inform and support decision making as hazard identification is needed (Valerio, 2012; Arvidson et al., 2010).

In order to assess the potential toxicological effects of onchidal from a predictive computational toxicology standpoint, the author subjected the two-dimensional molecular structure of onchidal (1) to an in silico QSAR and SAR computational analysis using open source software; Toxtree 3.1.0 (toxic hazard estimation by decision tree approach) and VEGA-QSAR 1.1.4 The toxicological endpoints predicted by the Toxtree computational SAR assessment system included the in vitro mutagenicity (Ames test) alerts by ISS, and carcinogenicity (genotox and nongenotox) and mutagenicity rule base by ISS. The QSAR models employed that are available by

TABLE 30.3 The two-dimensional molecular structure of onchidal was analyzed by Toxtree (3.1.0) and VEGA-QSAR (1.1.4) computational software.

Model	Alert or prediction
Toxtree: In vitro mutagenicity (Ames test) alerts by ISS	QSA10 Ames α,β-unsaturated carbonyls
Toxtree: Carcinogenicity (gentox and nongenotox) and mutagenicity rule base by ISS	QSA24 gen a,b unsaturated alkoxy, structural alert for genotoxic carcinogenicity
VEGA-QSAR: Mutagenicity (Ames test) CONSENSUS model	Mutagenic score = 0.28 representing low to good reliability
VEGA-QSAR: Carcinogenicity model (IRFMN/ Antares)	Noncarcinogenic two of six compounds in the training set which are highly similar to onchidal are noncarcinogenic. Four similar compounds in the training set are carcinogenic but their structural similarity is low

Toxtree uses a knowledge base of human expert rules encoded into a decision tree to perform a computational toxicology prediction on hazard for specific endpoints. VEGA-QSAR uses statistical and artificial intelligence approaches with chemical training sets that are used to construct the models (Benfenati et al., 2013). The data shown in the table indicate onchidal is theoretically predicted to be positive for mutagenicity in the Ames test using both SAR and QSAR, and negative for carcinogenicity using the QSAR approach, but fired an alert for carcinogenicity using the SAR method.

VEGA-QSAR were the mutagenicity (Ames test) consensus model 1.0.2 and carcinogenicity model (IRFMN/ Antares) 1.0.0 (Table 30.3).

These computational results show that onchidal is predicted to be of concern for bacterial mutagenicity (Ames test) in both the SAR and QSAR model systems. The structural alert that fired in Toxtree was an α,β-unsaturated alkoxy feature, and VEGA-QSAR used an ensemble of models of which the consensus was the compound would be mutagenic if tested. Toxtree indicated that the α,β-unsaturated alkoxy feature on the molecule is also alerting for carcinogenicity. However, VEGA-QSAR's specified modeling method did not predict the compound to be carcinogenic. The prediction indicated there are several compounds in the training set that are noncarcinogenic and two that are carcinogenic, thus the conflict amounted to an overall negative prediction due to the degree of similarity of onchidal with noncarcinogenic compounds.

Onchidal has anticholinesterase effects (Anadón and Martínez-Larrañaga, 1985). Tachycardia as a result of anticholinergic has been suggested to increase or lead to arrhythmias (e.g., with low-potency antipsychotics; Huffman and Stern, 2003), and this could call for further analysis using additional specialized QSAR/SAR models designed for alerting to cardiovascular toxicity.

Overall, these in silico/computational toxicology models, as well as many others, are growing in interest of enhancing hazard identification of compounds where there is human exposure and data are lacking. These models represent structure-based assessments using QSARs and SARs that if used properly may help predict with considerable precision the toxicological and adverse effects of many classes of organic substances (Valerio, 2009; Valerio et al., 2013). As with all methods, these models have some limitations, and in this regard, the main consideration with predictive modeling is the quality of the data used to build it. Advanced efforts have been focused on data integration techniques to combine empirical data with theoretical predictions (Valerio, 2012, 2013; Nigsch et al., 2011). Clearly, building in silico tools directed at understanding human adverse effects can be helpful for assessing safety and risk parameters that are difficult and time-intensive to determine in animals or acquire from well-controlled human studies. Thus, if properly validated, described, and made available for broad consumption, the in silico approach has the potential to be helpful (Valerio, 2013; Valerio and Choudhuri, 2012). We anticipate that as the use of new informatics tools, standards, and predictive technologies expand, these enabling methods will help build groundbreaking efforts to transform toxicology so that there is a better understanding of the toxic effects of at-risk substances such as onchidal and fasciculins.

30.6 Treatment

Treatment primarily involves supportive care and the i.v. administration of antivenom as soon as possible after neurological symptoms develop. The antivenom is indicated for all confirmed Eastern coral snake bites (Western, no antivenom) (Cameron, 2006). The South African polyvalent elapid antivenom covers all African species of elapids. The development of novel antivenoms and recombinant neutralizing antibodies demands, of course, the identification of the most relevant toxins in these venoms.

Antidotal therapy for the toxic effects of ChE inhibitors, such as onchidal and fasciculins used as potential chemical warfare agents, is directed toward blocking the effects of excessive ACh stimulation and reactivating the inhibited enzyme.

Atropine in sufficient dosage effectively antagonizes the actions at muscarinic receptor sites (Taylor, 2001). Atropine is used in adults at doses of 1−2 mg i.v. and in pediatrics at a dose of 0.05 mg/kg i.v.; doubling this dose every 5 min (DeLisle, 2006). Larger doses are required to get appreciable concentrations of atropine into the CNS. Atropine is virtually without effect against peripheral neuromuscular compromise.

Anticholinergic poisoning is managed by i.v. physostigmine, a reversible anti-ChE agent. Through its action at both central and peripheral cholinergic receptors, physostigmine reverses anticholinergic activity and ameliorates coma, delirium, and seizures that accompany severe toxicity. The initial i.v. or i.m. dose of physostigmine is 2 mg, with additional doses given as necessary.

The aforementioned action of the anti-ChE agents, as well as all other peripheral effects, probably can be reversed by reactivators of ChE such as pralidoxime (pyridine-2-aldoxime methyl chloride) (2-PAM) in the treatment of onchidal and fasciculin poisonings. The velocity of reactivation of phosphorylated AChE by 2-PAM varies with the nature of the phosphoryl group, following the same sequence as the order for spontaneous hydrolytic reactivation. The usual dosages of 2-PAM are as follows:

- In adults, 1−2 g i.v. over 15−30 min, may repeat in 1 h if necessary or start a drip at 500 mg/h;
- In pediatrics, 25 mg/kg i.v. over 15−30 min., follow with continuous infusion of 10−20 mg/kg i.v. (DeLisle, 2006).

30.7 Concluding remarks and future directions

The natural toxins onchidal and fasciculins behave as anti-ChE agents. Onchidal is an active, site-directed irreversible inhibitor of AChE, and fasciculins are proteinic AChE inhibitors that bind to a peripheral regulatory anionic site of AChE in a noncompetitive, irreversible manner. Both toxins could be used in chemical defense as potential warfare agents.

There is limited information about the toxicity, toxicokinetics, and toxicological properties of onchidal, and additional data are needed to make a health-effect-based risk assessment of the natural compound. Although fasciculins are much better known, data on toxicological properties and toxicokinetics will be of interest and useful for risk assessments. This is the case even though it is generally accepted that the toxicity of this proteinic poison occurs at very low doses.

Onchidal and fasciculins are interesting natural compounds, and it is difficult to predict their toxicity. As demonstrated in this chapter, predictive in silico approaches using computational models can be used to help assess the nonclinical toxicity of chemical agents. The quality of computational toxicology assessments relates to the quality of data used to derive predictions and appropriate expert interpretation of the data. Because the in silico approach is tailored toward filling data gaps, and enhancing decision making, the paucity of human data on onchidal and fasciculins makes a clear case for the use of in silico predictive technologies. The future of computational assessments seems to be in enabling techniques that would facilitate integrating theoretical predictions with empirical evidence in order to strengthen contemporary decision analysis. A major challenge will be to predict the military potential and overall human impact of these natural toxins since their biochemical affinity for enzyme inhibition depends upon the amount and duration of human exposure, and data about human exposure are lacking.

30.8 Disclosures

The authors declare that there are no conflicts of interest.

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Chapter 31

Cyanobacterial (blue-green algae) toxins

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31.1 Introduction

Cyanobacteria, also known as blue-green algae, comprise a diverse group of photosynthetic prokaryotic bacteria that proliferate in a wide range of freshwater and marine environments including ponds, lakes, reservoirs, and slow-moving streams, when the water is warm and nutrients and plenty of sunlight are available. Cyanobacteria are morphologically and physiologically diverse and perform photosynthesis through the green pigment, chlorophyll-a, and often produce accessory photosynthetic blue pigments, called phycobilins or phycocyanins. They also produce red to brown accessory photosynthetic pigments called phycoerythrins. Based on the levels of phycobilins and phycoerythrins, the colors of cyanobacteria vary between different shades of blue and green and, less commonly, brown to red. Since many genera of cyanobacteria have the ability to produce toxins, they are of toxicological significance, affecting aquatic organisms and terrestrial species including wildlife, livestock, pets, and even humans with ingestion of contaminated water or seafood (van der Merwe, 2015; Buratti et al., 2017; Kaur, 2019).

Cyanobacteria may be benthic or pelagic and, based on their morphology, they represent one of the most diverse prokaryotic phyla. Cyanobacteria have been divided into five different morphological sections including unicellular colonies with binary fission, unicellular colonies with multiple fission, multicellular colonies, multicellular colonies with differentiated cells, and branched multicellular colonies with differentiated cells (Schirrmeister et al., 2011). They form colonies often large enough to be seen with the naked eye and, when numerous, may change the color of the water or form scum at the water surface. An algal bloom is dense proliferations of cyanobacteria in a water system. Cyanobacteria blooms are often called blue-green algae. Algal blooms having the potential to cause harmful effects are called harmful algal blooms (HABs). Blooms can last from a few days to many months. They have been increasing in size and frequency in most regions and are generally induced by an overabundance of nutrients in the water, with the two most common nutrients being fixed nitrogen and phosphate from agricultural and industrial sources. Contributing factors are low circulation and higher water temperature (Hudnell, 2010; Gkelis and Zaoutsos, 2014; van der Merwe, 2015).

Many cyanobacterial species are capable of producing a wide range of toxins. Eutrophication and also other environmental factors increase bloom formation, including low turbulence, stagnant water conditions, higher pH values, and higher temperature (Bláha et al., 2009). The occurrence of a cyanobacteria bloom does not necessarily indicate that there is production of toxin. Multiple genotypes of cyanobacteria can exist in a single bloom, and some produce toxins while others do not. Under some conditions, even cyanobacteria genotypes or species that can produce toxins do not always produce toxins. The growth of the cyanobacterial species and their cyanotoxin production are influenced by many environmental factors including temperature, light intensity, pH, nutrients, salinity, ultraviolet radiation, wind, trace metals, and environmental pollutants. The toxins produced by cyanobacteria are also regulated by limitations of nutrients (nitrogen, phosphorus, and carbon), light, heat, oxidants, and other components. Toxin production is generally more common during warm weather and abundant sunlight, but can occur at any time of the year. The environmental conditions that trigger or inhibit production of cyanotoxins are not fully understood and remain an active area of research

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(Neilan et al., 2013; Boopathi and Ki, 2014; Szlag et al., 2015; van der Merwe, 2015). The production of toxin during HABs can be tremendously high and it has been documented that a HAB formed by microcystin-LR-producing *Microcystis aeruginosa* in a Kansas lake in 2011 produced toxin concentrations in the water of 126,000 ng/mL (van der Merwe et al., 2012). Such toxin concentrations in the water are highly significant taking into consideration that hepatotoxic effects in humans are possible following ingestion of 100 mL of water containing 20 ng/mL microcystin-LR (WHO, 2003). The spontaneous production of high concentrations of toxins in a natural environment, although somewhat sporadic, could make them accessible to harvesting for use as chemical weapons, requiring only simple equipment and low levels of technical capability. On the other hand, it is possible to obtain toxins from cyanobacteria produced in bioreactors, but this may require higher equipment investment and technical competence. Of toxins produced by cyanobacteria, saxitoxin or paralytic shellfish poison (PSP), which acts by blocking voltage-gated sodium channels, is listed as a Schedule 1 substance in the Chemical Weapons Convention. Other toxins produced by various genera of cyanobacteria may also pose credible threats based on their potency to produce deleterious effects, persistence in the environment, and widespread production in surface waters where they may be easily accessible (van der Merwe, 2015).

The cyanotoxins produced by freshwater cyanobacteria can be classified based on their origins, molecular structures, or adverse effects. They are generally grouped into major classes according to their toxicological targets, namely hepatotoxins (microcystins, nodularins, cylindrospermopsins), neurotoxins [anatoxin-a, anatoxin-a(s), saxitoxins], dermatotoxins (irritants), cytotoxins (aplysiatoxin, lyngbyatoxin-a), and endotoxins (irritants) (lipopolysaccharides) (Bláha et al., 2009).

31.2 Hepatotoxins

31.2.1 Microcystins and nodularins

31.2.1.1 Introduction

Microcystins are cyclic heptapeptides commonly produced by cyanobacteria genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Oscillatoria*, *Anabaenopsis*, and *Hapalosiphon*. They are called hepatotoxins because the liver is their primary target. Anthropogenic nutrient over-enrichment (eutrophication), but also other factors including expansion of intensive agriculture, rapid industrialization, and urbanization, seem to enhance the occurrence of microcystin-producing HABs in most regions. Microcystins have received increasing worldwide attention in the past decade because of their widespread and frequent occurrence, persistence in the environment, and their high potential to produce deleterious effects on human health.

Nodularins are mainly produced by *Nodularia spumigena* in brackish waters. They are similar to microcystins in structure and mechanism of toxicity and, for the purposes of this discussion, are considered equivalent to microcystins (Namikoshi et al., 1994; Chen et al., 2013a). In general, microcystin concentrations are correlated with cell density, even though high concentrations of cyanobacterial cells without toxin production are possible. The most common routes of human exposure to cyanotoxins are through the chronic and accidental ingestion of contaminated drinking water, inhalation or contact with the nasal mucous membrane, and dermal contact with toxins during recreational activities such as swimming, canoeing, or bathing. Other routes of exposure include consumption of contaminated vegetables, fruits and aquatic organisms, cyanobacterial dietary supplements, and the specific intravenous route caused by dialysis (Drobac et al., 2013).

31.2.1.2 Chemistry

Microcystins share a general cyclic heptapeptide structure which has the ability to resist physical and chemical factors (Fig. 31.1). The general structure of microcystins is cyclo-(-D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha), where Adda is (2S, 3S, 8S, 9S) 3-amino-9 methoxy-2,6, 8-trimethyl-10-phenyldeca-4,6-dienoic, D-MeAsp is D-erythro-b-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and X and Z are variable L-amino acids (Sivonen and Jones, 1999; Massey et al., 2018; Yang et al., 2018a, b). The ADDA side chain is consistent between microcystin variants and can be used to quantify microcystins independent of the variant type. Based on combinations of X and Z, the two variable L-amino acids, more than 100 microcystin variants with different degrees of toxicity have been isolated from cyanobacterial blooms. Among the different variants, the most common are microcystin-LR, microcystin-RR, and microcystin-YR, and the X and Z variable amino acids for microcystin-LR, microcystin-RR, and microcystin-YR are leucine (L), arginine (R),

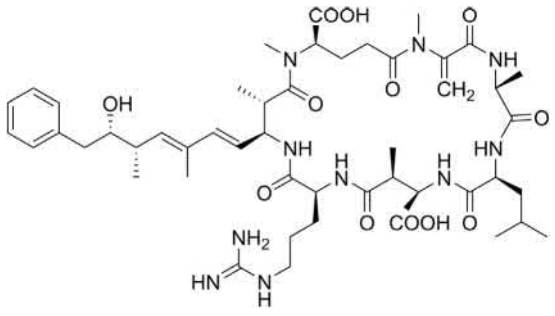

FIGURE 31.1 Microcystin chemical structure.

and tyrosine (Y) (Puddick et al., 2015; Yang et al., 2018a, b). Microcystin-LR is the most toxic, well studied, and abundant variant and is one of the most important algal toxins that has received worldwide attention (Zhou et al., 2013; Massey et al., 2018).

Microcystins are not actively secreted by cyanobacterial cells and therefore are strongly associated with cyanobacterial cells as long as the cells remain intact. However, microcystins may be released into the surrounding water after a bloom senesces or cyanobacterial cells are ruptured. Microcystins are stable, with a typical environmental half-life of 10 weeks. Under natural conditions, microcystins are resistant to physicochemical stresses, such as pH, temperature, and sunlight. However, the rate of breakdown is increased under direct sunlight, at high environmental temperatures ($>40^{\circ}C$), and at extremely low (<1) or high pH (>9) (Harada et al., 1996). Microcystin concentrations may be reduced by cooking in a microwave oven or by continuous boiling (Gutierrez-Praena et al., 2013). On the other hand, the concentration of free microcystins in muscle tissue from exposed fish may increase after boiling because of the release of phosphatase-bound microcystins (Zhang et al., 2010).

31.2.1.3 Toxic effects

The health effects of cyanotoxins are based largely on animal studies, usually mouse, rat, or pig, with some indirect data on humans obtained from forensic and epidemiological studies (Carmichael and Boyer, 2016; Greer et al., 2018; Massey et al., 2018). Only in a few cases have these studies been extended to chronic or teratogenic impacts on populations at risk (Meneely and Elliott, 2013). Our understanding of the toxicological effects of microcystins has been mainly based on microcystin-LR. The deleterious effects of other microcystin variants appear to be similar to those of microcystin-LR, but they differ in potency. Besides other factors, dose, potency, exposure route, duration of exposure, and intensity of exposure of cyanobacterial toxins employed in a particular laboratory animal species as well as interspecies variations in toxicokinetics and toxicodynamics can make the comparison with or extrapolating to the toxic effects of these toxins in other species challenging (Chen et al., 2018).

Exposure to microcystins represents a health risk to humans and many incidences of human exposure have been reported. The first confirmed human deaths from cyanotoxins occurred from intravenous exposure in a dialysis clinic in Brazil in 1996 (Jochimsen et al., 1998). Of 131 patients, 116 patients experienced visual disturbances, nausea, and vomiting. Consequently, 100 patients developed acute liver failure and this syndrome, now known as "Caruaru syndrome," resulted in 76 deaths. Of these, 52 deaths were a result of the dominant cyanobacterial toxins in the water supply reservoir, and it was concluded that the major contributing factor to death of the dialyses patients was intravenous exposure to microcystins (Carmichael et al., 2001).

The clinical effects of microcystin poisoning depend on the route of exposure, level of exposure, and the mixture of components involved in the exposure. In typical exposures of mammals to toxic blooms, low to mild exposure levels are associated with irritant effects, resulting in inflammatory responses in the skin, respiratory system, and gastrointestinal system. Higher exposure levels, particularly oral exposures, result in liver damage and, if the liver damage is severe, liver failure (Briand et al., 2003). The earliest detectable signs of liver damage include liver swelling and increased concentrations of liver enzymes such as alanine aminotransferase and aspartate aminotransferase in the blood. Early symptoms typically appear within minutes to hours of exposure and include inappetence, depression, and vomiting, followed by diarrhea, which may become extreme and hemorrhagic. Inappetence and depression become progressively worse. The final stages of lethal poisoning may be associated with recumbency and coma (van der Merwe et al., 2012; Kaur, 2019).

Microcystin-LR is a potential carcinogen for animals and humans, and is now classified as a potential class 2B carcinogen to humans. In addition to the liver, the testes has been considered as one of the most important target organs of microcystin-LR toxicity (Lone et al., 2015). Microcystins are one of the environmental carcinogens for the progression of colorectal carcinoma (Miao et al., 2016). Microcystins can also stimulate epithelial−mesenchymal transition (EMT) in colorectal cancer cells. During tumor progression, cancer cells acquire motility and an invasive property by regulating EMT that can lead to distant metastasis and are involved in embryonic development and cancer progression (Ren et al., 2017).

An important difference between exposures in aquatic organisms and terrestrial organisms is that exposures in aquatic environments often involve exposures to sublethal doses over days or weeks. In fish, exposures to microcystins cause cellular damage, particularly liver damage, similar to the effects seen in mammals (Tencalla and Dietrich, 1997). Mature fish are generally more resistant to microcystin toxicosis compared with juvenile fish. Although fish have the ability to avoid areas of accumulation of toxic algae, sublethal liver damage in fish is associated with accumulation of microcystins in fish food items such as mussels, snails, and zooplankton (Malbrouck and Kestemont, 2006). Fish are also susceptible to decreased water oxygen levels associated with the decay of algal scum, and this effect may also play a role in fish deaths (Ibelings and Havens, 2008).

Lower and higher concentrations of microcystin-LR-induced apoptosis and necrosis in lymphocytes,

respectively. In phagocytes, reorganization of the actin cytoskeleton, cell shrinkage, and filopodia disappearance were also observed, indicating immune system impairment (Rymuszka and Adaszek, 2013). Microcystin following epidermal exposure caused irritation of the skin. In cases of skin damage, faster penetration to deeper cell layers and absorption to the systemic circulation may be expected. Persistent toxic effects comprising of keratinocyte migration and cytoskeleton were only reported after longer exposures to the skin (Kozdeba et al., 2014).

31.2.1.4 Mechanism of action

Following ingestion and release from cyanobacterial cells, microcystins are absorbed into the portal circulation from the small intestine via bile acid transporters in the intestinal wall. Since the primary target cells for microcystins are in the liver, they are accumulated in hepatocytes via organic anion-transporting polypeptides (OATPs, OATP1B1, OATP1B3) in hepatocyte membranes. As a result of the abundance of OATPs expressed in the liver, the majority of ingested microcystin accumulates in hepatocytes. In addition to the liver, these cyanotoxins can affect other tissues including the kidneys, reproductive tissue, colon, and brain, that contain the appropriate OATPs (1A2, 1B1, 1B2, 1B3) (Carmichael and Boyer, 2016; Chen et al., 2016). OATP is also expressed in the blood−brain barrier, which may explain the neurological symptoms associated with human intoxication by microcystin in Brazil (Azevedo et al., 2002).

Microcystins are potent and irreversible inhibitors of protein phosphatases (PPs), mainly PP1 and PP2A. Inhibition of PPs leads to an increase in cell-phosphorylated protein load and subsequent deregulation of fundamental cellular processes and apoptosis. Microcystin-LR may also bind to ATP synthase, leading to hepatocyte apoptosis (Mikhailov et al., 2003). The microcystin-interacting PPs are ubiquitous and are found in all tissues and across species in mammals, plants, and bacteria. PPs reverse the active state of kinases through hydrolytic removal of the phosphoryl group from kinases. Inhibition of PPs also leads to changes in the cytoskeleton including hyperphosphorylation of cytokeratins, reorganization, disassembly of actin and microtubules, and ultimately disruption of cellular architecture (Huang et al., 2015; Zeng et al., 2015). Furthermore, PP2A inhibition also results in activation of the MAPK pathway (ERK1/2, JNK, p38) and apoptosis. Microcystin-LR impairs PP2A ability to bind tubulin and thus destabilizes the microtubules. The hepatotoxic effects of microcystin-LR proceed through alterations in cytoskeletal architecture and cell viability by MAPK activation, and hyperphosphorylation of p38, ezrin, and ERK1/2 (Komatsu et al., 2007; Kaur, 2019).

Microcystins produce toxicity mainly through oxidative stress, which was evident by marked alterations in cytotoxicity markers such as leakage of lactate dehydrogenase enzyme, lipid peroxidation, reactive oxygen species (ROS) generation, and antioxidant enzymes. Microcystin-LR also induces JNK activation, which affects enzymes involved in energy metabolism and mitochondrial dysfunction, ultimately resulting in apoptosis of hepatocytes and oxidative liver injury. Intracellular ROS production by microcystin-LR has also been shown due to a calcium-mediated loss of mitochondrial membrane potential. Microcystin-LR-mediated oxidative stress occurs with an increase in hydroxyl radicals and a subsequent induction of apoptosis-related genes p38, JNKa, and Bcl-2 in the liver of fish (Valério et al., 2016; Kaur, 2019). Calcium is known to be involved in the microcystin-mediated toxicity and may trigger mitochondrial dysfunction (Ding et al., 2001). In addition to calcium, ceramide is involved in microcystin-induced deleterious effects through regulation of the PP2A subunit, subcellular localization and inhibition, and cytoskeletal destabilization (Li et al., 2012). Chronic exposure to low-level microcystin-LR was found to inhibit fatty acid β-oxidation and hepatic lipoprotein secretion and promoted hepatic inflammation, leading to nonalcoholic steatohepatitis disease in mice (He et al., 2017).

Microcystins are capable of inducing deleterious effects in organs other than the liver including the kidneys, lungs, heart, and reproductive and immune systems (Massey et al., 2018). Oxidative stress and cytoskeletal disruption may interact with each other and jointly lead to apoptosis and renal toxicity induced by microcystins (Huang et al., 2015). Microcystins can cross the blood−brain barrier and blood−cerebrospinal fluid barrier and accumulate in the brain to induce neurotoxic effects, and neuroinflammatory and neurodegenerative disease due to the disruption of the mRNA levels involved in amino acid γ-aminobutyric acid (gabaminergic system), cytoskeleton, oxidative stress, and inhibition of PP1 and PP2A in the nerve cells (Li et al., 2012; Takser et al., 2016; Yan et al., 2017; Massey et al., 2018).

Microcystin-LR is classified as a possible class 2B carcinogen to humans. The ERK1/2 pathway, increased expression of protooncogenes namely cfos, c-jun, and c-myc in mouse, and p53 have been shown to be involved in microcystin-LR-induced tumor. Furthermore, microcystin-LR tumorigenesis was suggested to be regulated by NF-κB, interferon alpha (IFN-α), and tumor necrosis factor alpha (TNF-α) (Li et al., 2009; Christen et al., 2013; Valério et al., 2016). Microcystins may also produce alterations in expression of microRNAs in the liver to induce liver injury and influence liver tumor promotion (Yang et al., 2018a,b). Microcystins are associated

with the progression of colorectal carcinoma and can stimulate EMT in colorectal cancer cells (Miao et al., 2016; Ren et al., 2017).

Chronic exposure to low doses of microcystin can produce toxic effects on the male reproductive system due to a decrement of testosterone and testicular atrophy. Microcystins can lead to a decline in quality and viability of sperm, sperm abnormalities, and injury to the testes. Apoptosis can also be caused in Sertoli cells and Leydig cells (Ding et al., 2006; Chen et al., 2013b). Studies on the female reproductive system indicate that microcystins induced a direct reproductive toxicity resulting in decline of ovary weight, a decrease in primordial follicles, disturbance of estrus cycle, and decrease in serum progesterone (Wu et al., 2014). In cell culture models, microcystin-LR has been shown to induce Fas receptor, ligand, and NF-κB, which are important signals for apoptosis, and immune responses, and thus indicating its ability to modulate the immune system (Feng et al., 2011; Ji et al., 2011). Intraperitoneal administration of microcystin-LR in mice was demonstrated to increase thyroid hormones resulting in thyroid dysfunction. The glucose, triglyceride, and cholesterol metabolism were disrupted concurrent with symptoms of hyperphagia, polydipsia, and weight loss (Zhao et al., 2015).

31.2.1.5 Chemical warfare potential

Most microcystins are extremely toxic to freshwater bodies, marine ecosystems, and human health. Due to their capability to accumulate in aquatic species and food crops, they represent a health hazard in humans and animals through the food chain (Massey et al., 2018). Under favorable conditions including suitable nutritional levels and weather conditions, toxic concentrations can reach extremely high levels and represent an opportunity for the collection and storage of microcystins using widely available equipment and with minimal technical skill. The food supplies and water contaminated with microcystins represent significant human health risk. However, the major impact of such an event will be the cost associated with avoidance of exposure, including testing, water purification, and the procurement of alternative water and food sources (van der Merwe et al., 2012, van der Merwe, 2015). The most well-known occurrence of a harmful effect of microcystins is the first confirmed human deaths at a hemodialysis center following the use of contaminated dialysis water supplies (Azevedo et al., 2002). As a preventive measure to reduce risks caused by microcystins, the WHO recommends a provisional guideline value of 1 μg/L for microcystin-LR concentration in drinking water and a chronic tolerable daily intake of 0.04 μg/kg body mass per day for human consumption (Li et al., 2017). Although the potential efficacy of various chemoprotectants including antioxidant compounds and transport inhibitors to prevent or mitigate the toxic effects induced by microcystins has been investigated, there are no specific antidotes yet available for microcystin poisoning (van der Merwe, 2015; Guzmán-Guillén et al., 2017).

31.2.2 Cylindrospermopsin

31.2.2.1 Introduction

Cylindrospermopsin is an alkaloid cyanotoxin produced by several freshwater cyanobacteria genera, including *Cylindrospermopsis*, *Aphanizomenon*, *Anabaena*, *Lyngbya*, *Umezakia*, and *Raphidiopsis*. It is found worldwide in surface freshwaters and is a potential toxicant in drinking water supplies for a large human population, as well as animals (Guzmán-Guillén et al., 2013). It was first reported in 1979 after a hepatoenteritis outbreak occurred due to *Cylindrospermopsis raciborskii* bloom in the local drinking water supply in Palm Island, northern Queensland, Australia (Bourke et al., 1983).

31.2.2.2 Chemistry

Cylindrospermopsin is a polyketide alkaloid consisting of a tricyclic guanidine moiety combined with hydroxymethyluracil (Fig. 31.2). Due to its zwitterionic nature, cylindrospermopsin is a highly water-soluble compound. At present, there are five known structural variants of cylindrospermopsin, namely cylindrospermopsin, 7-epicylindrospermopsin, 7-deoxy-cylindrospermopsin, and the two recently characterized congeners, 7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetyl-cylindrospermopsin, which have been isolated from a Thai strain of *C. raciborskii* (Wimmer et al., 2014). Cylindrospermopsin is resistant to high temperatures, sunlight, and pH extremes. Unlike microcystins, cylindrospermopsin is often secreted from cyanobacterial cells into the surrounding water (Rucker et al., 2007). It bioaccumulates, particularly in organisms, in the lower level of the food chain, such as gastropods, bivalves, and crustaceans (Kinnear, 2010).

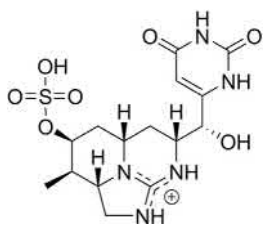

FIGURE 31.2 Cylindrospermopsin chemical structure.

31.2.2.3 Toxic effects

There are a number of worldwide reports on the impact of cylindrospermopsin exposure on human health. The toxin may produce a wide range of toxic effects. Cylindrospermopsin primarily targets the liver, but it is also a general cytotoxin that attacks the eyes, spleen, kidneys, lungs, thymus, heart, etc. (Guzmán-Guillén et al., 2017). A major outbreak of poisoning in humans with 148 cases occurred in 1979 near a reservoir on Palm Island, Queensland, Australia. The reservoir produced a dense bloom of cylindrospermopsin-producing *C. raciborskii* and the incident occurred a few days after treatment of the reservoir with copper sulfate to control the bloom. Finally, algal cell lysis and release of toxic cellular components occurred into the water. People in households connected to the reticulated water supply from the reservoir were affected with a syndrome that included liver and kidney damage, as well as severe gastroenteritis. Symptoms included hemorrhagic diarrhea, vomiting, fever, hepatomegaly, dehydration, electrolyte imbalances, acidosis, and hypovolemic shock. However, the potential role that copper sulfate water treatment could have played in the disease process remains uncertain. Intraperitoneal injection of extracts from *C. raciborskii* collected from the reservoir produced similar liver and kidney damage in mice (Hawkins et al., 1985). Cylindrospermopsin was also isolated from water used in dialysis in Brazil, which caused liver failure in dialysis patients. However, the role of cylindrospermopsin in the disease process was not clear because the water was also contaminated with toxic concentrations of microcystin (Azevedo et al., 2002).

C. raciborskii can cause mild skin irritation in some individuals, but the exact mechanism of action involved has not been elucidated. Although cylindrospermopsin outbreaks have been recently reported, there had been many instances of co-occurrence with other cyanobacterial toxins, making it difficult to confirm the relative contribution to symptoms. A similar outbreak, commonly known as "Barcoo fever," was reported in the Australian outback (Hayman, 1992). Pure cylindrospermopsin injected into tilapia caused progressive tissue damage over a period of 5 days in the liver, kidneys, heart, and gills (Gutierrez-Praena et al., 2013).

Administration of purified cylindrospermopsin by daily gavage in mice for 11 weeks increased the liver and kidney weight and alterations in hepatic and renal toxicity markers. Concomitant histopathological changes at the higher doses were also seen (Humpage and Falconer, 2003) Liver and kidney damage was consistently observed in laboratory rodents after exposure to acutely toxic doses of cylindrospermopsin-containing extracts. Typical liver pathology included lipid infiltration and necrosis, mostly in the periacinar region (Shaw et al., 2000). Dose-dependent DNA damage in mammalian cells has also been reported, which can be prevented by cytochrome P450 inhibitors, indicating an involvement of metabolic enzymes in progressing toxicity (Humpage et al., 2005). No reliable data on the cylindrospermopsin-mediated human carcinogenicity, genotoxicity, and reproductive/developmental toxicity are yet available (Kaur, 2019).

31.2.2.4 Mechanism of action

Cylindrospermopsin appears to be a molecule with a wide range of deleterious effects. Although the toxin primarily targets the liver, it is also a general cytotoxin that affects the eyes, spleen, lungs, thymus, heart, and lately the kidneys have been shown to be the more sensitive target of its toxicity (Guzmán-Guillén et al., 2017). Four sequential phases of hepatocyte damage were identified by a time series analysis, including protein synthesis inhibition, membrane proliferation, lipid infiltration, and necrosis. Kidney pathology included necrosis of the proximal tubules and protein accumulation in the distal tubules (Falconer et al., 1999). Studies using crude extracts reported higher potency and a wider range of effects compared with studies using purified cylindrospermopsin, indicating that other components also contributed to the adverse effects (Shaw et al., 2000).

Cylindrospermopsin is a potent inhibitor of protein synthesis in a concentration-dependent and irreversible manner, as confirmed both in in vivo and in vitro studies (Terao et al., 1994; Froscio et al., 2003), but the exact mechanism of action of the toxin has not been fully elucidated. A decrease in cylindrospermopsin toxicity by the administration of cytochrome P450 inhibitors has been reported, which suggests an alternative toxicity mechanism possibly through metabolite formation. Cylindrospermopsin has been shown to cause DNA fragmentation in vitro, and metabolic activation by a cytochrome P450 enzyme seems to be necessary for inducing this effect (Bazin et al., 2010). Recently, it has been shown that cylindrospermopsin induces upregulation of phase I biotransformation enzymes (CYP1A1, CYP1B1, ALDH1A2, and CES2) and phase II biotransformation enzymes (UGT1A6, UGT1A1, NAT1, and GSTM3) in human hepatoma HepG2 cells (Straser et al., 2013). In cell culture models, cylindrospermopsin has been shown to induce stress responses which resulted in the activation of the tumor suppressor protein p53 transcription factor (Bain et al., 2007).

Oxidative stress is one of the toxic mechanisms identified as being responsible for cylindrospermopsin toxicity. It is well known that cylindrospermopsin inhibits synthesis of glutathione, which is one of the major endogenous antioxidants that is produced by cells. In addition, cylindrospermopsin induced apoptosis in primary rat

hepatocytes and protein synthesis inhibition and increased ROS production were found to be involved in the cytotoxic effect elicited by the toxin (Lopez-Alonso et al., 2013). Cylindrospermopsin in subtoxic concentrations overexpressed proteins involved in DNA damage repair and transcription, including modifications of nucleosomal histones (Huguet et al., 2014). More recent experimental evidence using in vitro models of human bronchial epithelial cells showed that cylindrospermopsin is a potent cytotoxin causing significant inhibition of cell viability, which was accompanied by alterations in epithelial integrity and barrier function markers, along with activation of MAPK ERK1/2 and p38 (Kubickova et al., 2019).

31.2.2.5 Chemical warfare potential

Humans are more susceptible to the exposure to cylindrospermopsin as compared to other cyanotoxins, because up to 90% of total cylindrospermopsin is found outside the cyanobacterial cells (Rücker et al., 2007). The toxic effects of cylindrospermopsin are not yet fully established and additional data for hazard characterization purposes are required. It has been emphasized that research on various aspects including new emerging toxicity effects, the toxicity of analogs, or the potential interaction of cylindrospermopsin with other cyanotoxins, among others, remains very scarce (Pichardo et al., 2017). Despite the fact that the mechanisms of toxicity of cylindrospermopsin are not yet fully understood, ample evidence of its potential impact on human health exists, based on clinical cases, in vitro studies, and animal model studies (Poniedziałek et al., 2012). Owing to its increasing expansion worldwide and the increased frequency of its blooms, cylindrospermopsin is gaining increasing importance. The primary threats include contamination of drinking water supplies as well as food supplies.

31.3 Neurotoxins

31.3.1 Anatoxin-a

31.3.1.1 Introduction

Anatoxin-a is a very potent and fast-acting neurotoxin and is considered to be a major cyanotoxin of public health concern. It occurs worldwide in freshwaters and is produced by several genera of cyanobacteria, including *Anabaena*, *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Raphidiopsis*, *Arthrospira*, *Cylindrospermum*, *Phormidium*, *Nostoc*, and *Oscillatoria*. Exposures occur mainly through consumption of contaminated drinking water but have also occurred from recreational use of lakes and through contaminated dietary supplements (Osswald et al., 2007).

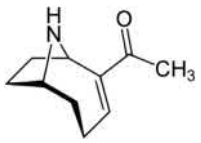

FIGURE 31.3 Anatoxin-a chemical structure.

31.3.1.2 Chemistry

Anatoxin-a is a bicyclic amine alkaloid (Fig. 31.3). It contains a homotropane scaffold that is derived from glutamic acid. Anatoxin-a is a chiral compound with asymmetric centers at C(1) and (5), and only one enantiomer has been reported from natural sources. The (+)-anatoxin-a enantiomeric form is naturally produced with a p*K*a of 9.4, indicating that it is mostly protonated under typical environmental pH conditions. Anatoxin-a is stable under sterile conditions but is susceptible to microbial biodegradation (Wonnacott and Gallagher, 2006). The half-life in a reservoir is reported to be 5 days under typical environmental pH conditions (Smith and Sutton, 1993). A structural analog, called homoanatoxin-a or methylene-anatoxin-a, has been isolated from *Oscillatoria formosa* (Skulberg et al., 1992). Small quantities of anatoxin-a are produced synthetically for use in acetylcholine receptor research.

31.3.1.3 Toxic effects

Anatoxin-a was originally called Very Fast Death Factor because of its rapid lethal effects, within 2–7 min, in laboratory mice. Acute deaths after exposure to anatoxin-a have been recorded in multiple species, including dogs, cattle, and wildlife, and interspecies differences in susceptibility to anatoxin-a have been observed even within animal types. Mallard ducks, for example, are more sensitive compared with ring-necked pheasants (Carmichael and Biggs, 1978). Anatoxin-a is rapidly absorbed from the gastrointestinal tract, as indicated by the rapidity of appearance of clinical effects after oral exposure. Clinical effects of poisoning may appear within minutes to hours after exposure and include loss of muscle coordination, muscle tremors and fasciculations, convulsions, and respiratory distress. The principal lethal effect is respiratory failure after loss of control over respiratory muscles (Osswald et al., 2007).

31.3.1.4 Mechanism of action

Anatoxin-a is an agonist of peripheral and central acetylcholine receptors, with a 100-fold selectivity for nicotinic receptors over muscarinic receptors. It binds to the acetylcholine receptor at the same position as acetylcholine (presynaptically and postsynaptically), causing sodium/potassium ion channels to open and induce a depolarizing blockade. Anatoxin-a is more potent than acetylcholine or

nicotine. Binding of anatoxin-a to the nicotinic acetylcholine receptors at neuromuscular junctions results in uncontrolled action potential propagation that manifests clinically as uncoordinated muscle contraction, muscle fatigue, and paralysis. The cholinesterase enzyme does not breakdown anatoxin-a, leading to persistent muscle stimulation (Wonnacott and Gallagher, 2006). Stimulation of nicotinic receptors in the cardiovascular system causes increased heart rate and blood pressure (Sirén and Feurstein, 1990). Stimulation of presynaptic nicotinic receptors in the central nervous system by anatoxin-a may also cause the release of neurotransmitters such as dopamine, which could further increase the susceptibility of postsynaptic receptors to overstimulation (Wonnacott and Gallagher, 2006). Central nervous system receptors are, however, less sensitive to anatoxin-a when compared with peripheral receptors.

31.3.1.5 Chemical warfare potential

Due to its high potency, fast action, and occasional availability of highly lethal concentrations of toxin during natural cyanobacterial blooms, anatoxin-a is potentially dangerous as a chemical warfare agent. Since anatoxin-a is vulnerable to rapid bacterial breakdown under nonsterile storage conditions, its intentional use is challenging without relatively sophisticated extraction and storage procedures (van der Merwe, 2015).

31.3.2 Anatoxin-a(s)

31.3.2.1 Introduction

Anatoxin-a(s) is a natural organophosphate analog produced by cyanobacteria in the genus *Anabaena*. The (s) in the name of anatoxin-a(s) refers to salivation, which is a characteristic sign of poisoning observed in laboratory rodents after exposure. Although anatoxin-a(s) has been less frequently reported, it was detected in animal toxicoses in Europe and the United States.

31.3.2.2 Chemistry

Anatoxin-a(s) is a cyclic *N*-hydroxyguanine with a phosphate ester moiety (Fig. 31.4) similar to typical synthetic organophosphate insecticides and chemical warfare nerve agents. No structural variants of anatoxin-a(s) have been described. Anatoxin-a(s) is susceptible to rapid degradation compared with other cyanotoxins (Matsunaga et al., 1989). The alkyl chain of anatoxin-a(s) (cyclic guanidines), which can be used as an intermediate in the total synthesis of anatoxin-a(s), was synthesized in both racemic and enantiomerically pure forms (Moura and Pinto, 2010). C-2, C-4, C-5, and C-6 of the anatoxin-a(s) are derived from the guanido carbon, C-5, C-4, and C-3 of L-arginine, respectively, and the three *O,N*-methyl groups originate from the tetrahydrofolate C_1 pool. *Erythro*-4-Hydroxy-L-arginine, a minor constituent of the cyanophyte, may be a precursor in the biosynthesis (Moore et al., 1992; Patocka et al., 2011).

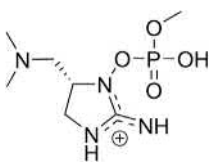

FIGURE 31.4 Anatoxin-a(s) chemical structure.

31.3.2.3 Toxic effects

Anatoxin-a(s) intoxication is characterized by severe cholinergic stimulation of both nicotinic and muscarinic receptors and clinical signs exhibited are indistinguishable from organophosphate poisoning. Muscarinic signs include salivation, lacrimation, urinary incontinence, and defecation, whereas nicotinic signs include muscle tremors and fasciculations, convulsions, and respiratory failure (Mahmood and Carmichael, 1986). The muscarinic effects of anatoxin-a(s) can be suppressed by atropine (Cook et al., 1990), but it is resistant to oxime reactivation due to the formation of an adduct on the acetylcholinesterase (AChE) enzyme (Hyde and Carmichael, 1991). Mice administered with *Anabaena flos-aquae* died within minutes due to respiratory arrest following convulsions. Prior to decreases in respiratory function, lowering of heart rate and blood pressure was also observed. As the inhibition of AChE enzyme was irreversible, surviving mice showed inhibition of erythrocyte cholinesterase for at least 8 days accompanied by twitching and fasciculations, indicating prolonged cholinesterase inhibitory action and toxic effects of anatoxin-a(s) at sublethal doses (Cook et al., 1991; Patocka et al., 2011; Miller et al., 2017).

31.3.2.4 Mechanism of action

Anatoxin-a(s) is a noncompetitive and irreversible inhibitor of AChE enzyme and, unlike the organophosphates, acts only on the peripheral nervous system in humans (Carmichael and Falconer, 1993). Similar to other organophosphate insecticides, anatoxin-a(s) is bioactivated via oxidative metabolism and ultimately binds to AChE enzyme. Acetylcholinesterase is present at all cholinergic synapses and is necessary for the inactivation of acetylcholine by hydrolysis. Inhibition of the enzyme therefore causes the acetylcholine level to build up at the receptor sites, triggering excessive stimulation of nicotinic and muscarinic receptors, resulting in persistent postsynaptic membrane depolarization (Cook et al., 1990). Since anatoxin-a(s) is a direct agonist at muscarinic receptors with indirect neuromuscular blockade, its effects can be blocked at least temporarily by atropine (Cook et al., 1989; Miller et al., 2017).

31.3.2.5 Chemical warfare potential

Similar to anatoxin-a, anatoxin-a(s) is a potential threat but its implementation will require relatively sophisticated procedures and equipment.

31.3.3 Saxitoxins

31.3.3.1 Introduction

Saxitoxins are often referred to as PSPs. Most human saxitoxin toxicoses have been associated with the ingestion of marine shellfish, which accumulate saxitoxins produced by marine dinoflagellates. Saxitoxins are also found in freshwaters and are produced by cyanobacteria in the genera *Anabaena*, *Aphanizomenon*, *Planktothrix*, *Cylindrospermopsis*, *Lyngbya*, and *Scytonema* (Wiese et al., 2010).

31.3.3.2 Chemistry

Saxitoxins are highly polar, nonvolatile, tricyclic perhydropurine alkaloids (Fig. 31.5) derived from imidazoline guanidinium. Various structural substitutions produce at least 57 analogs. Activity is mediated through positively charged guanidinium groups. Saxitoxins are heat-stable, particularly in slightly acidic environments, and are highly water-soluble. They are tasteless and odorless and are not destroyed by cooking (Trevino, 1998). Saxitoxins can bioaccumulate in freshwater fish such as tilapia (Galvão et al., 2009). Methods of synthetic saxitoxin production have been reported (Akimoto et al., 2013).

31.3.3.3 Toxic effects

The clinical presentation of saxitoxin poisoning varies depending on the level of exposure. High levels of exposure are usually related to ingestion of toxin-accumulating shellfish or fish, in association with toxin-producing dinoflagellate blooms (marine environments) or cyanobacterial blooms (freshwater environments). The lag time between exposure and the appearance of clinical signs is highly variable and can range from minutes to as long as 72 h. At relatively low exposure levels, moderate paresthesias, often described as a tingling sensation, are experienced around the mouth and extremities. Larger exposures lead to a spreading numbness of the mouth, throat, and extremities. High exposure levels may also cause acute muscle paralysis and respiratory failure (Garcia et al., 2004; Miller et al., 2017).

FIGURE 31.5 Saxitoxin chemical structure.

31.3.3.4 Mechanism of action

Saxitoxins are selective but reversible blockers of voltage-gated sodium channels. These toxins can cross the blood–brain barrier and cause sodium channel blockade in the central nervous system, contributing to paralytic effects (Wiese et al., 2010). They partially block L-type calcium channels, especially in the heart, and modify the voltage gating processes in potassium channels (Wang et al., 2003; Su et al., 2004). Therefore, early symptoms of a tingling sensation followed by paralysis and death usually occur due to suffocation or cardiac arrest (Miller et al., 2017).

31.3.3.5 Chemical warfare potential

Among cyanotoxins, saxitoxins only are listed in the Chemical Weapons Convention Schedule 1, where it is referred to as Agent TZ. Saxitoxins are also listed in the War Weapons List of the German War Weapons Control Act. There is a relatively high level of concern regarding saxitoxins as they are potent and stable, can be produced synthetically, and have been shown to be suitable for incorporation into ammunition. The regulatory control of saxitoxins gets rather complicated due to the ongoing need for its availability as an analytical standard in paralytic shellfish poisoning monitoring and risk management programs, and its use in medical research on voltage-gated sodium channels. Due to these requirements, saxitoxins are subjected to lower levels of restriction under the Chemical Weapons Convention when small quantities (5 mg or less) are to be used for diagnostic or medical purposes (van der Merwe, 2015).

31.4 Concluding remarks and future directions

Under favorable conditions for cyanobacteria proliferation, some cyanotoxins, in particular microcystins, can reach extremely high concentrations during HABs and these could be collected and stored with minimal technical sophistication and equipment. The use of such cyanotoxins with intention to contaminate water, food, and medical supplies represents a significant health and economic risk. Other cyanotoxins possessing high toxicity and infrequent availability during HABs such as cylindrospermopsin, anatoxin-a, and anatoxin-a(s) are potentially dangerous, but their intentional use is less likely due to their main limitations, such as unpredictable and occasional availability and relatively tedious extraction and storage procedures. The highest risk is represented by

neurotoxins with specific effects on the nervous system, such as saxitoxins, because they are listed in the Chemical Weapons Convention as a Schedule 1 compound. The listing of saxitoxins compared to other cyanotoxins is justified keeping in mind their high toxicity, stability, availability of synthetic methods for production, and suitability for incorporation into ammunition.

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Chapter 32

Chemical warfare agents and the nervous system

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32.1 Introduction

Chemical warfare has a long history. One of the earliest forms of chemical warfare involved the application of natural toxins to the tips of arrowheads and spears, used by early civilizations in Europe, Asia, South America, and Africa and described in Greek and Norse mythology (Mayer, 2008). The various types of chemical warfare agents (CWAs) are now listed as scheduled chemicals (Schedules 1, 2, and 3) in the Chemical Weapons Convention (Organisation for the Prohibition of Chemical Weapons, OPCW). The use of CWAs in modern warfare began in World War I (WWI, 1914–18), during which chlorine gas, mustard gas, and phosgene (primarily dermal and/or pulmonary irritants) were used. Mustard gas was again used during WWII. A number of organophosphorus chemicals, targeting the cholinergic nervous system, were synthesized as potential CWAs during the 1930s and 1940s and are often referred to as "nerve gases," or more appropriately "nerve agents." There remains widespread concern for threats from CWAs, in particular the organophosphorus nerve agents, reinforced by their military use in the Iran-Iraq War of 1980–88, by terrorists in Japan in the mid-1990s, sarin munitions in 2013 and April 2017 in the Syrian conflict, and the use of the highly toxic nerve agent VX in the murder of Kim Jong-un's half-brother.

Worldwide concern over the use of organophosphorus nerve agents was particularly heightened by the more widespread military events in Syria. As noted above, a documented large-scale use of organophosphorus nerve agents occurred in August of 2013 in the Ghouta area of Damascus, Syria. Sarin was employed via surface-to-surface rockets with a considerable number of casualties. Although there are conflicting reports on the numbers, at least 3600 people presented to medical facilities with neurotoxic signs and symptoms and 588 fatalities were recorded by the Violations Documentation Centre, which reports to the United Nations (Dolgin, 2013; Enserink, 2013; Patrick et al., 2013).

The Syrian government reportedly possessed a stockpile of over 1000 tons of chemical agents and precursors including sulfur mustard, sarin, and VX. Another documented attack occurred later in Khan Sheikhoun, Syria. There have also been reports of the use of chlorine gas in the Syrian conflict. These concerns were being mitigated by efforts of the OPCW-United Nations joint mission in Syria, which targeted the removal and/or destruction of all priority chemicals in Syria by June 30, 2014. The OPCW stated on September 4, 2014, that 96% of that country's declared stockpile of chemical weapons had been destroyed.

The different types of CWAs can affect one or multiple major organ systems with systemic exposure. This chapter focuses on the nervous system as a target for CWAs. A brief overview of the nervous system, highlighting some of the special features that often contribute to its unique sensitivity to CWAs (and toxicants in general) is provided. Specific information on the effects of selected CWAs on the nervous system are discussed for illustration.

32.2 Overview of the nervous system

The nervous system can be divided into two major structural divisions: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of

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the brain and spinal cord, covered by three "membranes"—the meninges. The outermost layer is the dura mater. The PNS consists of all parts of the nervous system that lie outside of the dura mater. The PNS can be classified based on functionality as the autonomic and somatic nervous systems. The autonomic nervous system, which can be further divided into the sympathetic, parasympathetic, and enteric divisions, regulates visceral functions and various processes that are not under voluntary control, while the somatic nervous system controls voluntary skeletal muscle activity. Maintenance of homeostasis and proper communication within and among these various components of the nervous system is critical for physiological coordination and normal functioning of the whole organism.

There are two basic types of cells in the nervous system: neurons ($\approx 10\%$ of the total number of cells) and glia ($\approx 90\%$ of the cells, including astrocytes, oligodendrocytes, NG2 cells, microglia, satellite cells, and Schwann cells). Neurons are highly specialized cells optimized for the conduction of electrical impulses to other nerve cells and to innervated tissues such as glands and muscles. In all neurons there exists a soma (cell body), dendrites (smaller branching structures), and an axon (electrical impulse-conducting fiber) that terminates in multiple endings and gives rise to specialized areas, that is, the presynaptic terminals. The junctional region between the presynaptic terminal and its target (innervated) cell is the synapse, where intercellular communication occurs, mediated by extracellular signal molecules (e.g., neurotransmitters) released by the presynaptic terminal. Anatomically, neurons can be classified into three basic types based on extensions of the dendrites and axon: unipolar, bipolar, and multipolar. Functionally, neurons can be characterized into afferent neurons (sensory neurons), efferent neurons (motor neurons), and interneurons. Afferent neurons conduct electrical impulses from tissues to the CNS, efferent neurons carry electrical impulses from the CNS to the tissues, and interneurons transmit information between neurons.

Despite having a markedly higher density in the nervous system, the glial cells were historically considered to provide metabolic and physical support for the neurons: this has been shown to be an oversimplification of the function of these types of cells. For example, astrocytes aid in the maintenance of homeostatic conditions of the extracellular fluid surrounding neurons by removing excess potassium ions. Microglia participate in phagocytosis following neuronal damage/degeneration. Oligodendrocytes and Schwann cells (found in the CNS and PNS, respectively) form the lipid-rich myelin layers around axons, which effectively enhance conduction down the axon and prevent the spread of impulses between juxtaposed axons. It is now recognized that glial cells play many important roles as key regulators of many aspects/processes associated with development, aging, and toxicology/pathology, such as synaptogenesis, reactive gliosis, immunoreactivity, and homeostasis of neurotransmitters (Aschner et al., 2002; Allen, 2013; Verkhratsky et al., 2014; Pajarillo et al., 2019).

Neurons are excitable cells undergoing dynamic phases of depolarization and repolarization as they mediate extracellular signaling. As with other cells, neurons maintain a resting potential across the plasma membrane, typically -60 to -75 mV, relative to the extracellular fluid. When fully excited, the membrane potential in a neuron exceeds a threshold voltage, which triggers a series of events that result in the initiation and propagation of an action potential along the axon to the terminal region. Each action potential begins with a rapid reversal of voltage from the negative resting potential to a positive potential (depolarization phase) and ends with an almost equally rapid change to a negative potential (repolarization phase). Once initiated, the action potential is self-propagated to the terminal region of the axon in an "all-or-none" (without decrement) fashion. When an action potential reaches the presynaptic terminal, voltage-gated Ca^{2+} channels open in response to depolarization, resulting in extensive influx of Ca^{2+} ions, which then trigger presynaptic vesicle exocytosis. The synaptic vesicles fuse with the plasma membrane and release their contents (neurotransmitters and other cosignals) into the synaptic cleft. Electrical impulses are thus transformed into chemical signals and thereby transduced from one (presynaptic) neuron to another (postsynaptic) neuron, muscle cell, or autonomic end organ through synaptic transmission.

Extracellular communication in the nervous system is typically mediated through synaptic transmission via the release of neurotransmitters and their subsequent binding to specific receptors. The transmitter−receptor interaction then generally elicits changes in ion channel permeability and/or second messenger formation in the innervated cell. Neurotransmitters can also interact with receptors located on the presynaptic terminal (either *autoreceptors* that are activated by the same transmitter or *heteroreceptors* that are activated by a different transmitter, released by a different neuron) to regulate presynaptic cell function, often by modulating further neurotransmitter release. Termination of synaptic neurotransmission is dependent upon the removal of neurotransmitter molecules from the synaptic cleft by either *enzymatic degradation* (e.g., by acetylcholinesterase in cholinergic neurons) or by *reuptake* into the presynaptic terminal (e.g., by a monoamine transporter in serotonergic neurons).

Classical neurotransmitters in the nervous system can be classified into the amino acids (e.g., glutamate, aspartate, GABA, glycine), the monoamines (e.g., catecholamines, serotonin, histamine), and acetylcholine. Adenosine triphosphate (ATP) and nitric oxide are

nonclassical neurotransmitters that often mediate cotransmission with other classical transmitters. Neurotransmitters typically mediate synaptic transmission in an anterograde fashion, that is, the transmitter molecules are released from a presynaptic terminal to diffuse across the synaptic cleft and bind to receptors on postsynaptic neurons or other types of cells to mediate unidirectional synaptic transmission.

In the last three decades, lipid metabolites known as endocannabinoids (eCBs) have gained attention as global neuromodulators, with the potential to influence a wide array of physiological and pathological processes (see reviews of Pacher and Kunos, 2013; Senst and Bains, 2014; Kendall and Yudowski, 2017). The eCBs are derived from membrane lipids that can modulate synaptic function in a *retrograde* fashion, that is, they are released by a postsynaptic cell but influence the activity of a presynaptic neuron. Anandamide (AEA) and 2-arachidonylglycerol (2-AG) are the two most prominent eCBs (i.e., endogenous lipid metabolites that directly bind/activate cannabinoid receptors). In the CNS, eCB signaling is mediated primarily by cannabinoid type 1 (CB1) receptors, a putative membrane transporter of eCBs, and enzymes involved in both synthesis (diacylglycerol lipase and *N*-acylphosphatidylethanolamine-specific phospholipase D) and inactivation (fatty acid amide hydrolase, FAAH and monoacylglycerol lipase, MAGL) of eCBs. Unlike classical neurotransmitters that are synthesized and stored in synaptic vesicles, the eCBs are synthesized "on demand" from arachidonic acid in the membrane lipids of neurons. Upon depolarization, the eCBs are synthesized and released from a postsynaptic neuron and then diffuse through the synapse to bind/activate CB1 receptors on the presynaptic terminal. The other major cannabinoid receptor subtype, CB2, primarily resides on immune cells, for example, activated microglia (Carlisle et al., 2002; Maresz et al., 2005), but eCBs may also modulate nervous system function by activating CB2 receptors on neurons (Morgan et al., 2009; García-Gutiérrez et al., 2012; Li and Kim, 2015). Endocannabinoid synthesis and release can also be stimulated in a receptor-mediated fashion by activation of the Gq protein-coupled metabotropic glutamate receptors and muscarinic M1 and M3 receptors. Termination of eCB signaling appears to require cellular reuptake via a carrier-mediated transport process. Following cellular reuptake, eCBs are enzymatically degraded. FAAH is distributed throughout the brain and appears to be primarily responsible for AEA hydrolysis, while MAGL is the primary enzyme involved in 2-AG degradation. Both FAAH and MAGL are intracellular enzymes and inhibition of either/both can lead to increases in eCB levels (Liu et al., 2013, 2015; Buntyn et al., 2017; Carr et al., 2014, 2017; Shin et al., 2019).

Depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) are two forms of synaptic plasticity mediated by eCBs. In classical DSI, postsynaptic neurons release eCBs, which in turn decrease GABA release in presynaptic GABAergic neurons and disinhibit postsynaptic cell activity. In DSE, the eCBs reduce presynaptic glutamate release from glutamatergic neurons, leading to a net suppression of postsynaptic cell activity.

The nervous system is complex both anatomically and functionally. The extensive networks mediating intracellular and extracellular signaling make it highly vulnerable to disruption by many toxicants. Moreover, the nervous system possesses additional unique features that can contribute to its higher sensitivity to toxicants, including CWAs.

32.2.1 Special features of neurons and high energy demand

As noted above, neurons are composed of a cell body (or soma), extended, branching dendrites, and an elongated axon. Because of this structural complexity, most neurons have relatively large cell volumes compared to other cell types. The unique spatial features of neurons often require the synthesis of a large amount of proteins in the soma and their transport over long distances through processes of axonal transport. The Nissl substance, found exclusively in soma of neurons and formed by clusters of ribosomal complexes, synthesizes the large amount of proteins needed for these cells. Unlike other cells, neurons are particularly excitable, continuously generating and transmitting electrical signals along their axons and chemical signals across the intercellular synapses. The neurons, therefore, have an absolute requirement for a continuous and abundant supply of energy to support the need for synthesis of large amounts of proteins and neurotransmitters, for transport of proteins, cellular organelles, and cytoskeletal components to distant parts of the cell, and to maintain and restore ionic gradients affected by cycles of depolarization/repolarization.

The nervous system depends heavily on aerobic glycolysis to generate energy. The demand for oxygen and glucose is so high and sustained that even a brief interruption of oxygen and/or glucose may result in severe adverse consequences as there is essentially no reserve in the brain. The high oxygen requirement is associated with approximately 20% of total cardiac output going to the brain. Toxic agents that interrupt the supply of oxygen (e.g., carbon monoxide) or the utilization of oxygen (e.g., cyanide) can, therefore, produce catastrophic cellular and functional damage in the nervous system.

The nervous system is a lipid-rich environment. As noted above, many elongated axons in the CNS and PNS are insulated by concentric layers of myelin that facilitate conduction of electrical impulses. Because of the density of the lipids, myelinated axons can be more sensitive to lipophilic neurotoxicants. Moreover, axons in the adult CNS have very limited ability to regenerate after injury, at least partially because of myelin-associated inhibitory factors from oligodendrocytes and "glial scars" from reactive astrocytes. In contrast, axons in the PNS have a greater potential to regenerate and regrow after injury under the differential environment provided by Schwann cells.

32.2.2 Blood–brain barrier

Most of the adult brain and spinal cord is anatomically separated from the circulation by a continuous lining of specialized endothelial cells whose apposed surfaces form tight junctions. This, aided in part by associated glial cells, constitutes a *blood–brain barrier* (BBB), largely limiting passage of substances that are large and hydrophilic, unless there is an active transport mechanism for facilitating transfer across the cellular membrane. The BBB plays a pivotal role in protecting the CNS from various chemical challenges, especially larger, hydrophilic toxicants. In humans, this BBB is incompletely developed at birth, and even less so in premature infants. The barrier also does not exist in certain brain regions, for example, the area postrema, hypophysis, hypothalamic regions, pineal body, and the supraoptic crest. The deficiency of the BBB in these areas in the adult brain, and the incomplete BBB in the developing brain, can be important in differential sensitivity to chemicals. With many toxicants that are relatively hydrophobic (e.g., organophosphorus nerve agents) however, the BBB has little role in uptake and neurotoxicity. The integrity of the BBB can be compromised under certain conditions, for example, anesthesia, stress, metabolic and/or pathological disorders (e.g., adrenocorticoid hypertension, meningitis), leading to higher sensitivity to some neurotoxicants (see Kandel et al., 2013 for an extensive overview of nervous system structure and function).

32.3 Types of neurotoxicity

Based on the respective location of the original "lesion" elicited by a neurotoxicant, neurotoxic responses can be classified as neuronopathy, axonopathy, myelinopathy, or transmission-associated toxicity. *Neuronopathy* involves injury/death of neurons, with subsequent degeneration and loss of dendrites, axons, and myelin sheath (when present). Such neuronal degeneration is irreversible and can result in an encephalopathy with global dysfunction, dependent upon the neuronal population(s) affected. *Axonopathy* can be thought of as a "chemical transection" at some point along an axon, such that the distal segment degenerates but the soma remains intact. The critical difference between central and peripheral axonopathies is the ability of the axon to regenerate and reinnervate the "target" cell over time. A common, early clinical indicator of axonopathy is loss of peripheral sensations (e.g., glove and stocking paresthesia) and disruption in fine, distal motor control. *Myelinopathy* is a chemical-induced demyelination (i.e., loss of the myelin sheath) or intramyelinic edema (i.e., separation of the concentric layers of the myelin). Remyelination typically occurs with a much thinner myelin sheath and shorter intermodal distances than present before the toxic insult. *Neurotransmission-associated toxicity* typically occurs in the absence of any initial morphological change, but is related to toxicant-induced *neurochemical* changes within the synaptic region. Toxicants can disrupt neurotransmission by affecting presynaptic processes including neurotransmitter synthesis, transport, and/or storage in synaptic vesicles, neurotransmitter release, reuptake or degradation, by binding to presynaptic and/or postsynaptic receptors, by affecting ion flux in the presynaptic or postsynaptic cell, or by other mechanisms influencing signal transduction.

Neurotoxicity can also be classified based on the functions affected, for example, dysautonomia when the autonomic nervous system is disrupted, neuromuscular disorders when somatic motor neurons or the neuromuscular junction is affected, sensory neuropathy when sensory receptors or afferent sensory neurons are the primary targets, or cognitive deficits and mood/mental disorders when higher brain/cognitive functions are affected.

32.4 Selected chemical warfare agents that affect the nervous system

As briefly reviewed above, the nervous system possesses some unique anatomical, biochemical, and physiological characteristics that can contribute to its relatively higher sensitivity to xenobiotics, including CWAs. Many chemicals are potential weapons of mass destruction, including but not limited to organophosphate nerve agents, cyanides, mustards, arsenicals, and the natural products botulinum toxin and ricin. CWAs can adversely affect the nervous system either directly [i.e., the toxicant interacts with a receptor inside the nervous system (a molecular initiating event)] or indirectly (i.e., the disruption of nervous system function by a molecular initiating event outside the nervous system), leading to acute and/or chronic adverse neurological consequences.

32.4.1 Organophosphorus nerve agents

The organophosphorus nerve agents are widely recognized as CWAs and are among the most lethal CWAs ever developed. They are classified as either "G" or "V" series agents. The "G" agents including tabun (*O*-ethyl-*N*,*N*′-dimethylphosphoramidocyanidate; GA), soman (*O*-pinacolyl methylphosphonofluoridate; GD), sarin (isopropyl methylphosphonofluoridate; GB), and cyclosarin (cyclohexyl methylphosphonofluoridate; GF) are volatile and relatively nonpersistent, whereas the "V" agents such as VX (*O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonofluoridate) are less volatile and relatively more persistent. Both the United States and the Soviet Union stockpiled many of these agents for military use during the Cold War. However, the first documented military use of nerve agents was not until the Iran-Iraq War of 1980−88. During this conflict, Iraqi troops employed sarin-containing munitions against both military personnel and civilians, confirmed by detection of trace amounts of a breakdown product, isopropyl methylphosphonic acid, in soil samples taken from a Kurdish village in northern Iraq (Macilwain, 1993). Subsequently, sarin was used in two separate terrorist attacks in Japan by a cult named *Aum Shinrikyo* (Divine Truth). The first attack on June 27, 1994, occurred in a residential neighborhood in Matsumoto, resulting in 56 hospitalizations and 7 deaths. The second attack on March 20, 1995, occurred in several subway cars in Tokyo, resulting in 796 hospitalizations and 12 deaths (Yanagisawa et al., 2006).

Among the victims of the sarin attack in Tokyo, Japan, 111 individuals were characterized as severely or moderately injured on admission to hospital on the day of the attack and exhibited miosis (99%), headache (75%), respiratory distress (63%), nausea (60%) and vomiting (37%), eye pain (45%), blurred or dim vision (40% and 38%, respectively), and seizures (approximately 3%) (Okumura et al., 1996). Changes in psychomotor functioning, visual perception and sustained attention, learning and memory, and mood (Yokoyama et al., 1998; Murata et al., 1997) were observed in 18 survivors, evaluated 6 − 8 months later. Depressive behaviors were reported in survivors between 6 months and 7 years after the attack. This included those who had not presented with symptoms of acute toxicity at the time of the incident (Araki et al., 2005). Yamasue and colleagues (2007) observed smaller regional white matter volumes and diffuse bilateral disruption of white matter integrity 5−6 years after the attacks. Miyaki and colleagues (2005) reported deficits in attention and gross motor speed 7 years after the attacks. Hoffman et al. (2007) reported that digit span, finger-tapping, and computerized posturography tests showed statistically significant differences between victims of the Tokyo attacks and controls, while the most consistent consequence was post-traumatic stress disorder.

Organophosphorus toxicants, including nerve agents, elicit acute toxicity primarily via inhibition of acetylcholinesterase, disrupting homeostatic regulation of acetylcholine-mediated cholinergic signaling in the CNS and PNS. Acetylcholine is synthesized in the presynaptic terminal by choline acetyltransferase using choline and acetyl CoA as substrates. Once synthesized, acetylcholine molecules are stored in the synaptic vesicles. Upon depolarization, the vesicles containing acetylcholine fuse with the presynaptic membrane and release acetylcholine into the synapse. The diffusible acetylcholine molecules then interact specifically with cholinergic (muscarinic and nicotinic subtypes) receptors on postsynaptic cell membranes to mediate signal transduction. Muscarinic receptors are G protein-coupled receptors that modulate the formation of second messengers [e.g., cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), and inositol triphosphate (IP3)]. Five muscarinic receptor subtypes (M1−M5) have been identified and cloned. M1, M3, and M5 subtypes are coupled to phospholipase C (PLC) via G_q and increase the formation of DAG and IP3 upon activation, whereas M2 and M4 subtypes are coupled to inhibition of adenylyl cyclase via G_i and decrease the formation of cAMP. Nicotinic receptors are ligand-gated ion channels that generally increase Na^+ influx upon stimulation. Acetylcholine can also bind to presynaptic muscarinic and nicotinic receptors to modulate the further release of acetylcholine in a negative (or sometimes positive) "feedback" manner. Acetylcholine in the synapse is degraded very rapidly by acetylcholinesterase into choline and acetate. The choline molecules are transported back into the presynaptic terminal through a process known as *high-affinity choline uptake*, the rate-limiting step in acetylcholine synthesis. Acetylcholine, therefore, has only a transient opportunity to activate muscarinic or nicotinic receptors, either postsynaptically or presynaptically. Inhibition of acetylcholinesterase prevents the normally efficient breakdown of acetylcholine in the synapse, leading to acetylcholine accumulation and persistent/prolonged stimulation of cholinergic receptors, which in turn elicits signs and symptoms of cholinergic toxicity.

Many of the classical signs of cholinergic toxicity associated with organophosphate poisoning involve the PNS. The well-recognized cholinergic signs of excessive secretions (salivation, lacrimation, urination, and defection, abbreviated by the acronym SLUD) and other sensitive changes, for example, miosis, are the result of acetylcholinesterase inhibition and muscarinic receptor activation in peripheral tissues (Espinola et al., 1999). Other common signs of cholinergic toxicity, for example, muscle fasciculations, involve overstimulation of nicotinic receptors in peripheral tissues (Costa, 1988). However, the most debilitating effects of anticholinesterase exposures involve the CNS (Pope, 2006). Lethality from

organophosphate intoxication is typically due to depression of brainstem respiratory control centers, compounded by excessive airway secretions and dysfunction of diaphragm and intercostal muscles. Rickett and colleagues (1986) reported that one of the first signs of nerve agent-induced respiratory distress in cats was disruption of the normal firing of respiratory-related neurons in the pons medulla, followed by changes in airflow, diaphragm contraction, diaphragm electromyogram (EMG), and phrenic nerve activity. Spectral analysis of diaphragm EMG indicated that the functional integrity of the diaphragm in guinea pigs following soman exposure (15 μg/kg, s.c.) was not sufficiently compromised to produce respiratory distress despite signs of fatigue (Chang et al., 1990). These studies suggested that respiratory distress following nerve agent exposure is mainly due to disruption of central cholinergic signaling. Bajgar et al. (2007) reported that acetylcholinesterase activity in the pons medulla (i.e., the site of respiratory control centers) was more extensively inhibited by soman, sarin, and VX than acetylcholinesterase in many other brain regions.

While tremors can be elicited by excessive activation of nicotinic receptors in the PNS, whole body tremors can result from extensive acetylcholinesterase inhibition in the CNS, with activation of muscarinic receptors in the basal ganglia being of prominent importance (Espinola et al., 1999). With severe intoxications, seizures can be elicited that lead to irreversible neuropathology (Shih et al., 1991; Kadar et al., 1995; Dekundy et al., 2001, 2007). Shih and McDonough (1999) demonstrated that all five of the classic nerve agents (i.e., cyclosarin, sarin, soman, tabun, and VX) were capable of inducing seizures at lethal dosages. In the case of VX, however, the latency of seizure development was three to five times longer than with the other nerve agents. Soman-induced seizures occurred in rats only when cortical acetylcholinesterase inhibition was over 65% (Tonduli et al., 2001). Anticholinergics (e.g., atropine and biperiden) and benzodiazepines (e.g., diazepam and midazolam) were able to terminate nerve agent-induced seizures in guinea pigs when administered within 5 min of seizure onset, but a higher dose of each was typically required to terminate seizures induced by soman compared to other nerve agents (Shih et al., 2003).

Prolonged seizure activity (i.e., *status epilepticus*) following nerve agent exposure has been shown in several animal models to cause neuropathological lesions that are associated with long-term deficits in cognitive function and other neurobehavioral endpoints. Moderate to severe neuropathological lesions were reported in 70% of sarin- (Kadar et al., 1995) and 98% of soman-treated (McDonough et al., 1995) rats, when seizures lasted 20 min or longer. Pathological lesions were primarily noted in hippocampus, piriform cortex, and thalamus, but later progressed to other brain regions, for example, amygdala. Soman-induced *status epilepticus* in rats resulted in significant neuronal loss and neurodegeneration, in particular with GABAergic interneurons, in the basolateral nucleus of the amygdala. Moreover, this neuropathology was associated with increased anxiety-like behavior measured in the open-field test and the acoustic startle response, 14 days later (Prager et al, 2014). Spatial memory performance deficits in the Morris water maze were directly related to the severity of hippocampal lesions in soman-treated rats. Interestingly, no memory impairment was observed below a threshold of 15% neuronal loss (Filliat et al., 1999). Impaired contextual and cued fear conditioning (fear-based learning and memory) were observed in soman-treated rats and mice that experienced seizures and neuronal degeneration in different brain regions (Moffett et al., 2011; Coubard et al., 2008). These mice also showed anxiety-like behaviors measured in unconditioned fear tests (light/dark boxes and elevated plus maze). Deficits in cognitive function measured in a novelty test, the brightness discrimination task (Myhrer et al., 2005), and the stone maze (Raffaele et al., 1987) were reported in rats following soman-induced seizures associated with neuropathological lesions.

Nerve agents such as soman and other organophosphorus anticholinesterases can induce behavioral changes linked to anxiety- and depressive-like behaviors in animal models, at dosages that do not elicit seizures. A sublethal dosage of soman ($0.6-0.8 \times LD_{50}$) triggered anxiety-like behavior (measured in the open-field test and elevated plus maze) in guinea pigs (Mamczarz et al., 2010). Acute intoxication with diisopropylfluorophosphate (DFP, a prototype organophosphate and structural analog of sarin) in rats increased immobility and decreased active swimming behavior in the forced swim test (Wright et al., 2010).

DFP (9 mg/kg, i.m.) in atropine- and pyridostigmine-pretreated rats led to seizures and delayed (4−72 h) apoptotic neuronal cell death in the hippocampus, cortex, amygdala, and thalamus as shown by Fluoro-Jade staining, with reduced NeuN immunoreactivity at 24 h after dosing (Li et al., 2011). Interestingly, neuregulin isoforms (NRG-EGF, NRG-GGF2) reduced both neuronal injury and apoptosis in this model (Li et al., 2012). Moreover, a time-course study indicated that astrocytic immunoreactivity (GFAP) increased in a brain regional manner, with GFAP in hippocampus and piriform cortex increasing from 1−8 h, decreasing out to 24 h, and then showing a marked increase from 3 to 7 days after dosing. The results suggested that glial activation may be an early precedent of neuropathology in this model (Liu et al., 2012). A more recent study (Li et al., 2015) indicated that neuregulin-1 reduced microglial activation after DFP exposure. Microarray analysis suggested a reduction in proinflammatory cytokines including IL-1B and IL-6.

Flannery et al. (2016) and Hobson et al. (2019), using a similar DFP-rat model, studied the time course of immunohistochemical markers of neuroinflammation [GFAP and ionized calcium-binding adapter molecule 1 (IBA1)], along with PET imaging of the 18-kDa mitochondrial membrane translocator protein (TSPO). Neuroinflammation was noted in hippocampus and cortex from 3 days, peaking at 7 days and evident out to 21 days after dosing, and importantly was correlated with earlier seizure intensity. These and other results (Sisó et al., 2017; Guignet et al., 2019) confirmed a likely role for neuroinflammation in the delayed and persistent sequelae following OP anticholinesterase-induced seizures and acute cholinergic toxicity.

Chen et al. (2011) reported serotonergic-related changes in affective behaviors measured in the elevated plus maze, the Vogel's conflict test, the novelty-suppressed feeding test, and the forced swimming test in adolescent rats exposed to the common OP insecticide chlorpyrifos (10−160 mg/kg/day for 7 days, s.c.). Repeated exposure to another OP insecticide, methamidophos, in the drinking water at levels that did not elicit systemic toxicity induced depressive-like behavior in adult mice (Lima et al., 2009). Malathion exposure has also been reported to be associated with depressive-like behavior in rodents (Ramos et al., 2006; Brocardo et al., 2007; Acker et al., 2009).

Anxiety and depression have also been reported in humans following organophosphate intoxication. A number of epidemiological studies have reported long-term neuropsychological or neuropsychiatric sequelae, such as anxiety and depression, with past anticholinesterase intoxication or exposure (Savage et al., 1988; Rosenstock et al., 1991; McConnell et al., 1994; Steenland et al., 1994; Yokoyama et al., 1998, 2002; Wesseling et al., 2002; Colosio et al., 2003; London et al., 2005; Roldan-Tapia et al., 2006). A cross-sectional survey conducted among 761 individuals, representing 479 farms in northeastern Colorado between 1992 and 1997, found that exposure to pesticides, in particular organophosphates, sufficient to cause self-reported poisoning symptoms was associated with a more than five times higher rate of depression, independent of other known risk factors (Stallones and Beseler, 2002). A mini review of 11 studies on depression in relation to pesticides revealed increased odds ratios (ranging from 2 to 6) for developing depression or other psychiatric disorders (Freire and Koifman, 2013). Wesseling et al (2010) reported an increased incidence of depression and anxiety, along with other psychological or psychiatric deficits, in workers exposed to organophosphates that had received medical attention 1−3 years prior to the study.

Thus, central actions of acute or repeated exposures to organophosphorus anticholinesterases can be critical in both acute and long-term neurological sequelae. While disruption of cholinergic neurotransmission is a hallmark of acute organophosphate poisoning, substantial evidence indicates disruption of noncholinergic signaling can contribute to the ultimate long-term outcome. Shih and coworkers (1991) reported that seizures elicited by soman were initially sensitive, but later resistant, to the antimuscarinic antidote atropine. Activation of glutamate signaling in limbic regions, for example, hippocampus, appeared particularly important in maintaining organophosphate-induced seizures (Lallement et al., 1991). These investigators observed a 78% increase in extracellular glutamate in the CA3 region of the hippocampus within 30 min of seizure onset, and a more robust early increase (180%) followed by a decline but more sustained increase in the hippocampal CA1 region after 50 min of seizure onset in soman-treated rats. Sparenborg and colleagues (1992) reported that the NMDA antagonist MK-801 (30, 100, or 300 μg/kg, i.m.) arrested soman-induced seizures in a dose-dependent manner, suggesting that NMDA receptor activation plays a critical role in the spread and persistence of nerve agent-induced seizures. Moreover, NMDA receptor antagonists blocked seizures elicited by some organophosphorus insecticides (dichlorvos and chlorfenvinphos; Dekundy et al., 2001, 2007). In contrast, it is reported that higher striatal dopamine and GABA levels were correlated with the severity of soman toxicity, seizure intensity, and epileptiform bursting (Cassel and Fosbraey, 1996; Jacobsson et al., 1997). The dopamine D1-like antagonist SCH23390 completely blocked soman-induced seizures (Bourne et al., 2001). The activation of noncholinergic signaling pathways, therefore, appears to participate in the expression of organophosphate toxicity, and brain regional differences in the activation of noncholinergic signaling may be important in the ultimate neurotoxic outcome.

More than 100,000 United States military personnel participating in the 1991 Gulf War were potentially exposed to low levels of cyclosarin and sarin following the destruction of a munitions storage facility at Khamisiyah, Iraq, in March 1991 (Smith et al., 2003). Roughly 30% of Gulf War veterans later reported various neurological and other symptoms collectively referred to as Gulf War illnesses (GWI), characterized by impaired cognition, depression, insomnia, ataxia, disorientation, joint/muscle pain, extremity paresthesias, fatigue, headaches, and respiratory, gastrointestinal, and dermatologic complaints (Haley et al., 1997; Steele et al., 2015; White et al., 2016). Deficits in motor dexterity and visuospatial abilities (Proctor et al., 2006) and a twofold increase in the incidence of brain cancer-related deaths (Bullman et al., 2005) were reported in these veterans. In addition, a reduction in the overall volume of white matter and an enlargement of the lateral ventricles were observed in the brains of some veterans (Heaton et al., 2007).

An extensive review (RAC, 2008) found a causal relationship between exposure to anticholinesterases (pyridostigmine and/or pesticides) and GWI, while a later Institute of Medicine review (IOM, 2010) concluded there was insufficient information to confirm that association. The fact that pyridostigmine was *intentionally* taken (suggested dose was 30 mg three times a day) and thus constituted a relatively high-level exposure compared to many other potential environmental chemical exposures is important. A number of studies published since 2010 continue to provide support for a role of pyridostigmine in GWI. For example, Steele and coworkers (2015) found an association between pyridostigmine use and increased odds for GWI in those veterans having genetic variants of butyrylcholinesterase (the "sister" enzyme of acetylcholinesterase) with low enzymatic activity. White and colleagues (2016) concluded that exposure to pesticides and/or to pyridostigmine was associated with neurological symptoms of GWI in Gulf War veterans potentially exposed to sarin and cyclosarin. In a rat model of GWI with prior pyridostigmine exposures, lipopolysaccharide challenge led to increased C-reactive protein levels compared to controls, attenuated cholinergic neurochemical responses, and impaired behavioral responses in contextual and fear-based learning paradigms (Macht et al., 2019). Thus, the causal relationship between anticholinesterase exposures including pyridostigmine and development of GWI remains a viable hypothesis supported by substantial evidence.

GWI is regarded as a chronic multisymptom illness or CMI (IOM, 2013). Treatment strategies for relatively nonselective, multisymptom clusters are being actively evaluated. The cause or causes of GWI have been extensively evaluated and remain uncertain, but more effective treatments may be realized in the absence of a mechanistic explanation for these conditions. Although exposure to the organophosphorus nerve agents, organophosphorus pesticides, and the carbamate anticholinesterase pyridostigmine have been suspected of playing some role in the neurological dysfunction in GWI (Brimfield, 2012; Sullivan et al., 2018; White et al., 2016), the evidence overall has been insufficient to firmly establish such relationships.

Reviews of medical records for Gulf War veterans and published field accounts revealed no clinical indications of classic, acute organophosphate intoxication (Riddle et al., 2003). Again, the lack of evidence for acute cholinergic signs in affected individuals, coupled with the very high sensitivity of acetylcholinesterase to inhibition by these toxicants and thus to cholinergic toxicity, may be a clue that noncholinergic mechanisms are important in the etiology of GWI.

Neuroinflammation has been implicated in the development of a variety of neurological conditions (Schwartz and Deczkowska, 2016; Hobson et al., 2019). A number of studies suggest a role for neuroinflammation in the etiology of GWI (O'Callaghan et al., 2015; Coughlin, 2017; Shetty et al., 2017; Locker et al., 2017; Carreras et al., 2018; Michalovicz et al., 2019). A noncholinergic signaling pathway that may participate in inflammation and other anticholinesterase-mediated effects is the eCB system. Endocannabinoid signaling participates in a variety of processes including thermoregulation, food intake, immunomodulation, perception (hearing, color, vision, taste), cognition (long-term potentiation, short-term memory), and motor function (locomotor activity, proprioception, muscle tone). Of particular importance in cholinergic toxicity, eCBs inhibit the release of a number of neurotransmitters (Gifford and Ashby, 1996; Gessa et al., 1997; Levenes et al., 1998; Miller and Walker, 1995; Cadogan et al., 1997; Cheer et al., 2004). Several studies have suggested that eCBs tonically inhibit acetylcholine release in hippocampus via CB1 receptors, that is, agonists reduce while antagonists increase the release of acetylcholine (Gifford and Ashby, 1996; Gifford et al., 2000; Tzavara et al., 2003; Degroot et al., 2006; Gessa et al., 1997; Kathmann et al., 2001). Interestingly, acetylcholine release in the striatum is unaffected by eCB agonists/antagonists or by deletion of the CB1 gene in mice (Gifford et al., 2000; Kathmann et al., 2001). Thus, differential effects of eCBs on acetylcholine release in different brain regions may be important.

Endocannabinoid-mediated inhibition of GABA and glutamate release from presynaptic neurons via DSI and DSE may also play an important role in the expression of organophosphate toxicity. As mentioned above, higher levels of striatal GABA and limbic glutamate levels have been correlated with more severe signs of organophosphate toxicity (Lallement et al., 1991, 1992; Cassel and Fosbraey, 1996; Jacobsson et al., 1997). Furthermore, the $GABA_A$ receptor antagonist bicuculline is proconvulsant when directly administered into limbic regions but is anticonvulsant when microinjected into the striatum (Turski et al., 1989, 1991). Thus, changes in GABAergic and glutamatergic signaling and their modulation by eCBs could have regional-specific effects on neurological function after organophosphate intoxication.

While eCBs can regulate the release of acetylcholine and other neurotransmitters, organophosphorus toxicants may also affect eCB signaling, either indirectly through acetylcholinesterase inhibition (and neuronal depolarization/activation of M1/M3 receptors) or directly through binding to cannabinoid receptors and/or inhibiting cannabinoid metabolizing enzymes (Quistad et al., 2001, 2002, 2006; Segall et al., 2003; Nallapaneni et al., 2006, 2008). Chlorpyrifos oxon, paraoxon, and DFP inhibit CB1 receptor binding and the activities of FAAH and MAGL in vitro, with chlorpyrifos oxon being more potent. In

vivo, DFP inhibited FAAH but had little effect on MAGL or CB1 binding (Nallapaneni et al., 2008). Liu and colleagues (2013) reported that both chlorpyrifos and parathion (the parent compounds of chlorpyrifos oxon and paraoxon, respectively) inhibited FAAH and MAGL in vivo, with more extensive inhibition of FAAH by both compounds. Extracellular levels of eCBs in rat hippocampus were increased by both chlorpyrifos and parathion at dosages that inhibited >80% of hippocampal acetylcholinesterase activity.

Pharmacological manipulation of eCB signaling has been shown to affect both acute and long-term toxicity of organophosphates. The mixed CB1/CB2 receptor agonist WIN 55,212-2 (1.5 mg/kg, i.p.) reduced acute paraoxon toxicity with a single dose, but increased toxicity if paraoxon was given after repeated WIN 55,212-2 dosing (daily for 7 days; Nallapaneni et al., 2006). Liu and Pope (2015) showed increased cholinergic toxicity in rats exposed to either chlorpyrifos oxon or paraoxon and then challenged with the CB1 receptor antagonist/inverse agonist AM251. Farizatto and coworkers (2017) reported that paraoxon reduced synapsin IIb levels and elicited oxidative damage to proteins (4-hydroxynonenal-protein adducts) in hippocampal slices in vitro, which were reversed by the FAAH inhibitor AM5206. Moreover, preexposure to AM5206 in rats blocked paraoxon-induced seizures, reduction in synapsin IIb, and oxidative changes in the hippocampus and cortex. Acute DFP toxicity was reduced by WIN 55,212-2, as well as by inhibitors of FAAH and MAGL (URB597 and URB602, respectively), and the eCB reuptake inhibitor (AM404; Nallapaneni et al., 2008). As noted before, acute DFP exposure induced changes in the forced swim test in rats used extensively in tests for antidepressant efficacy. The combination of URB597 and URB602 reversed some DFP-induced changes in the forced swim test (Wright et al., 2010). Interestingly, the CB1 receptor antagonist/inverse agonist AM251 decreased parathion-induced toxicity in rats while having no influence on functional signs of chlorpyrifos (Liu et al., 2013). Thus, eCB signaling may play differential roles in toxic outcome of organophosphates based on the modulation of different neurotransmitter systems, possibly related to the time of onset and the duration of the neurotoxic response.

A number of studies have reported that young animals are generally higher in sensitivity to the acute toxicity of organophosphorus insecticides (Benke and Murphy, 1975; Pope et al., 1991; Moser, 2011; Padilla et al., 2000; Pope and Liu, 1997; Zheng et al., 2000). Interestingly, based on acetylcholinesterase inhibition and functional/behavioral changes, young rodents can actually be less sensitive to repeated anticholinesterase exposures, due to the differential rates of recovery of acetylcholinesterase activity after inhibition by an organophosphorus toxicant (Chakraborti et al., 1993). With lower level, repeated exposures to the pesticide chlorpyrifos in postnatal rats [0.5−1.0 mg/kg/day from postnatal day (PND) 10−16], eCB metabolism (by inhibiting FAAH) was affected in dosages that in most cases did not affect acetylcholinesterase activity and was associated with increased brain and peripheral tissue anandamide and 2-arachidonoyl glycerol levels and changes in anxiety-like behaviors (Carr et al., 2014, 2017; Buntyn et al., 2017).

Relatively less is known regarding the neurochemical and toxicological consequences of organophosphorus nerve agents in young individuals. Wright and coworkers (2016) reported that rats at PND 7, 14, 21, and 70 were more sensitive than at PND 28 and 42 to lethality from subcutaneous exposure to tabun, sarin, soman, and cyclosarin, but no age-related differences in sensitivity were observed with VM, VX, or Russian VX. Wright et al. (2017) also reported higher sensitivity to inhalation exposures to sarin in PND 14 rats, and again, with lowest sensitivity in the adolescent rats (PND 28). Miller et al. (2015) showed that PND 21 rats exposed to $1.2 \times LD_{50}$ soman that developed status epilepticus also had decreased brain acetylcholinesterase activity, especially in the basolateral amygdala. Soman-induced seizures in PND 21 rats were also associated with fear-learning deficits and increased anxiety at 30 days post-exposure, and reduced volume of the amygdala and hippocampus at 30 and 90 days despite no signs of neuronal degeneration in the brain at 1 or 7 days. Meanwhile, Scholl et al. (2018) reported that seizure severity was age-dependent: PND 14 rats exposed to sarin or VX showed no EEG seizure activity, while seizure activity increased in severity as a function of age in PND 21, 28, and 70 rats.

While we and others have focused on the role of endocannabinoids in modulation of organophosphorus anticholinesterase toxicity, an important issue is the increasing legal medical and recreational use of phytocannabinoids (in marijuana) in the United States and other countries. As the psychoactive phytocannabinoids can interact with the same cannabinoid receptor signaling pathways as the endocannabinoids, there is concern for how eCB signaling may be disrupted by cannabis use, in children or in adults, and how that could influence the sensitivity to an organophosphate through disruption of endocannabinoid-mediated neuromodulatory mechanisms (Nallapaneni et al., 2006; Liu and Pope, 2015; Farizatto et al., 2017). Further, the classical neuromodulatory role of CB1 receptor activation by endocannabinoids and phytocannabinoids (e.g., THC) influences synaptic signaling by modulating neurotransmitter release. In the presence of acetylcholinesterase inhibition, reducing further acetylcholine release can putatively reduce cholinergic toxicity. In contrast, activation of CB2 receptors has little to do with regulating neurotransmitter release but can play an

immunomodulatory role that could be important in some later responses to anticholinesterase exposures (Haugh et al., 2016; Mastinu et al., 2018). Moreover, THC as well as the nonpsychoactive phytocannabinoids, for example, beta-caryophyllene, can activate CB2 receptors while the endocannabinoid-like metabolites oleoylethanolamide and palimitoylethanolamide activate PPAR alpha receptors (Guzmán et al., 2004; O'Sullivan, 2007; Lindsey et al., 2019). As PPAR activation can also modulate inflammatory processes, all of these actions have the potential to modulate neuroinflammation that may be important in the later neurological complications of an acute anticholinesterase intoxication. With a number of studies implicating neuroinflammation in the delayed and persistent neurological sequelae following anticholinesterase intoxication, both endocannabinoids and phytocannabinoids may modulate the progression of delayed neuroinflammatory processes. These neuroinflammatory pathways may have importance in the ultimate outcome of acute organophosphate intoxication and in the etiology of GWI.

Organophosphorus anticholinesterases have the potential to markedly disrupt nervous system function, eliciting a broad range of acute and long-term effects. While acetylcholinesterase has been considered as the primary target of these toxicants, and downstream changes in cholinergic signaling have been the main focus in understanding mechanisms and exploring countermeasures, noncholinergic pathways following acetylcholinesterase inhibition may play a prominent role in the ultimate chronic outcome following organophosphate exposure.

32.4.2 Cyanides

Cyanides have been used for their toxic potential since ancient Roman times. Cyanogen chloride, cyanogen bromide, and hydrogen cyanide are important cyanide-containing compounds of potential use as CWAs. The use of cyanides in chemical warfare was not realized until WWI. Due to their high volatility, however, these compounds rarely achieve lethal atmospheric concentrations except in enclosed spaces (Lee, 1997). Hydrogen cyanide was used for mass extermination of prisoners in concentration camps by the Nazis during WWII. Other forms of cyanide-containing compounds have also been used for malicious purposes. For example, potassium cyanide was illicitly placed in capsules of Extra Strength Tylenol, leading to seven deaths in the Chicago area in 1982 (Dunea, 1983).

Signs of cyanide exposure include agitation, dizziness, headache, and mental confusion followed by cardiac disturbances, loss of consciousness, respiratory distress, seizures, and death. While cyanide exposure is often fatal, there are reports of long-term effects with sublethal intoxications. Finelli (1981) described a 30-year-old male, 1 year after an attempted suicide with cyanide, who developed choreiform movements in his extremities and impairment in the movement of his left hand. Similarly, Carella et al. (1988) reported a 46-year-old woman who drank a beverage poisoned with cyanide and later developed a dystonic posture of the mouth and tongue that was deviated to the right and twisted. Computed tomography scans showed that both individuals had lesions in the basal ganglia.

Although there are a number of reports on the neuropathological consequences of cyanide exposure including necrotic lesions in the cerebellar gray matter of dogs (Haymaker et al., 1952) and demyelinating lesions in the corpus callosum and optic nerves of rats (Lessell, 1971), its neurobehavioral sequelae have received relatively little attention. D'Mello (1986) reported that a single exposure to sodium cyanide (4 mg/kg, s.c.) impaired swimming ability in guinea pigs. Mathangi and Namasivayam (2000) demonstrated that repeated exposure to sodium cyanide (2 mg/kg/day $\times$ 30 days, i.p.) impaired T-maze performance in rats, along with a reduction in dopamine and 5-hydroxytryptamine levels in the hippocampus.

The primary mode of action for cyanide-induced toxicity is inhibition of cytochrome oxidase, the terminal enzyme of the electron transport chain. Inhibition of cytochrome oxidase leads to cytotoxic hypoxia, with a shift from aerobic to anaerobic metabolism, a decrease in ATP synthesis, and an increase in lactic acid production (Way, 1984). The CNS is particularly sensitive to the toxic effects of cyanide due to both extremely limited anaerobic metabolic capacity and high energy dependence. Ikegaya and colleagues (2001) showed that the inhibition of cytochrome oxidase activity following oral administration of potassium cyanide (10 mg/kg) in rats was highest in brain compared to other organs. Thus, not only is the CNS particularly sensitive to cyanide-induced hypoxia and energy deficit, but the target enzyme itself in the CNS may be more sensitive to inhibition by cyanide compared to enzyme in other tissues.

Oxidative stress may also play a critical role in cyanide-induced toxicity. Potassium cyanide (7 mg/kg, s.c.) decreased the activities of catalase, glutathione peroxidase, and superoxide dismutase in mouse brain (Ardelt et al., 1989). Potassium cyanide also stimulated the formation of reactive oxygen species and increased levels of malondialdehyde in a number of neuronal cell types including cerebellar granule cells (Gunasekar et al., 1996), primary cortical cells (Li et al., 2002), and rat pheochromocytoma (PC-12) cells (Kanthasamy et al., 1997). Johnson and colleagues (1986) proposed that a rise in intracellular Ca^{2+} levels following potassium cyanide exposure was responsible for the formation of reactive oxygen species. In support of this, Gunasekar and

colleagues (1996) reported that removal of Ca^{2+} from the culture medium blocked the formation of reactive oxygen species in cerebellar granule cells exposed to potassium cyanide (100 μM). Moreover, antioxidants (Muller and Krieglstein, 1995), Ca^{2+} channel blockers (Johnson et al., 1987), cyclooxygenase-2 (COX-2) inhibitors (Li et al., 2002), as well as NMDA receptor antagonists (Gunasekar et al., 1996) and phospholipase A2 inhibitors (Kanthasamy et al., 1997) all blocked the formation of reactive oxygen species in neuronal cell lines exposed to potassium cyanide.

Cyanide-induced cell death may involve selective activation of apoptosis or necrosis in different brain regions or neuronal populations. Mills and colleagues (1999) reported that cyanide induced cell death in mouse brain via apoptosis in the cortex, but through necrosis in the substantia nigra. Similarly, Prabhakaran et al. (2002) reported that cyanide elicited cell death via apoptosis in primary cortical cells but by necrosis in primary mesencephalic cells. While potassium cyanide (400 μM) increased the formation of reactive oxygen species in both cell types, the rates of formation and the nature of the reactive oxygen species varied. Furthermore, catalase and superoxide dismutase decreased the formation of reactive oxygen species in cortical cells but not in mesencephalic cells. These findings suggest that the selective vulnerability of different neuronal populations to cyanide may be related to differences in their susceptibility to oxidative stress.

Dopaminergic neurons are highly sensitive to oxidative stress, possibly due to dopamine auto-oxidation to quinones and other reactive oxygen species (Basma et al., 1995; Ben-Shachar et al., 1995). Kanthasamy and colleagues (1994) reported that mice repeatedly exposed to potassium cyanide (6 mg/kg, twice a day for 7 days, s.c.) had a reduced number of TH-positive cells, indicating a loss of dopaminergic neurons in the substantia nigra. Approximately 30% of the cyanide-treated mice exhibited decreased locomotor activity and akinesia, which were alleviated by the administration of L-DOPA (100 mg/kg, i.p.). Cassel and Persson (1992) reported that levels of striatal dopamine and its metabolite HVA were rapidly decreased in rats exposed to sodium cyanide (20 mg/kg, i.p.). However, the in vivo synthesis of dopamine, measured as the rate of L-DOPA accumulation after neuronal decarboxylase inhibition, was increased in these rats. Kiuchi and colleagues (1992) noted that perfusion of sodium cyanide (2 mM) into rat striatum produced a transient but marked increase in DA release. In addition, dopamine D1 and D2 receptor binding in the striatum was decreased in rats after the administration of sodium cyanide (2 mg/kg, i.p.) (Cassel et al., 1993). Given that Parkinson's disease is a condition characterized by the selective loss of dopaminergic neurons in substantia nigra and the depletion of striatal dopamine levels (German et al., 1989), these findings suggest that cyanide exposure may lead to the development of parkinsonian-like signs or symptoms.

Both glutamatergic and GABAergic neurons may be involved in the development of seizures after cyanide exposure. There is a strong correlation between whole-brain Ca^{2+} levels and cyanide-induced seizures in mice (Johnson et al., 1986). Patel and colleagues (1992) demonstrated that NMDA receptor activation was responsible for the rise in intracellular Ca^{2+} levels, contributing to cyanide-induced toxicity in cultured hippocampal neurons. Yamamoto and Tang (1996, 1998) reported that cyanide-induced seizures in mice were blocked by MK-801 (2 mg/kg, s.c.) and the morphological changes observed in cerebrocortical neurons exposed to potassium cyanide (1 mM) were blocked by 2-amino-7-phosphonoheptanoic acid (AP7; 1 mM): both are selective NMDA receptor antagonists. Persson and colleagues (1985) reported that striatal GABA levels were decreased in rats exposed to sodium cyanide (20 mg/kg, i.p.), and these decrements were associated with an increased susceptibility to seizures (Cassel et al., 1991). Similarly, Yamamoto (1990) reported that whole-brain GABA levels were markedly decreased and Ca^{2+} levels were increased in cyanide-treated mice exhibiting seizures. All these findings suggested that increased glutamatergic activity and/or decreased GABAergic activity may contribute to the development of seizures following cyanide exposure.

32.4.3 Sulfur mustard

Sulfur mustard (2,2′-dichlorethyl sulfide; HD) is a classical blister agent and an effective incapacitating chemical. It was first used as a CWA in WWI during a German attack on British troops at Ypres, Belgium, in 1915. Subsequently, it has been used in a number of military conflicts, including the Iran-Iraq War of 1980–88 (Marshall, 1984). During this conflict, Iraqi troops employed sulfur mustard against both military personnel and civilians. Approximately 40,000 victims of sulfur mustard have been documented among the Iranian and Kurdish populations (Hay, 2000; Khateri et al., 2003).

Sulfur mustard exerts local actions on the eyes, respiratory tract, and skin followed by systemic actions on the nervous, cardiac, gastrointestinal, and hematopoietic systems (Dacre and Goldman, 1996). A moderate exposure to sulfur mustard can cause blisters, conjunctivitis, erythema, lacrimation, nausea, and respiratory inflammation; whereas a severe exposure can cause blindness, bronchitis, bronchopneumonia, corneal damage, leucopenia, seizures, and death. Sulfur mustard is a strong alkylating agent that reacts with thiol, amino, carboxyl, hydroxyl, and primary phosphate groups in DNA and other macromolecules. Alkylation of DNA activates chromosomal

poly(ADP-ribose) polymerase (PARP), reducing the intracellular supply of NAD^+ and thus inhibiting glycolysis and causing cell death (Papirmeister et al., 1985). More recently, it was suggested that oxidative stress also may play a critical role in sulfur mustard-induced toxicity (Naghii, 2002). Jafari (2007) demonstrated that high dosages of sulfur mustard (> 10 mg/kg, i.p.) decreased the activities of catalase, glutathione peroxidase, glutathione S-transferase, and superoxide dismutase in rat liver and brain, leading to impairment of antioxidant defense systems.

There is limited information available on the effects of sulfur mustard on the CNS. The majority of the victims exposed to sulfur mustard during the Iran-Iraq War seeking medical treatment at European hospitals had anxiety, confusion, headache, and lethargy, with the potential of these neurological symptoms related to indirect effects (Balali-Mood and Hefazi, 2006). A cohort study conducted on 495 Iranian civilians revealed that individuals exposed to sulfur mustard exhibited a significantly higher incidence of depression, anxiety, hostility, and obsessive-compulsive behavior compared to age-matched counterparts 20 years after exposure (Roshan et al., 2013). Lethal dosages of sulfur mustard (> 10 mg/kg, s.c.) caused hyperexcitability followed by unsteadiness of gait, muscular weakness, and seizures in dogs (Lynch et al., 1918). Philips and Thiersch (1950) reported that lethal dosages of 2,4,6-tris(ethylenimino)-*S*-triazine (> 125 mg/kg, i.p.), which has an ethylenimine moiety analogous to sulfur mustard, caused similar effects in mice. Although the mechanism responsible for these effects is unknown, it is interesting to note that sulfur mustard induced acetylcholinesterase activity in neuroblastoma cell cultures, suggesting that cholinergic signaling potentially plays a role in its effects on the CNS (Lanks et al., 1975).

Brimfield (2012) suggested that recent studies provided evidence that sulfur mustard metabolism by NADPH-cytochrome P450 reductase could be important in the context of mixture exposures such as those that occurred in the Gulf War setting. In essence, carbon free radicals formed by this reaction could inhibit electron transport, with potential for oxidative stress exacerbation in the nervous system and other tissues. While sulfur mustard is typically thought of as a blister agent, clinical and experimental data suggest that it has the potential for eliciting both acute and long-term neurotoxic effects and may interact in a complex manner with multiple chemical coexposures.

32.4.4 3-Quinuclidinyl benzilate

3-Quinuclidinyl benzilate (QNB) (1-azabicyclo[2.2.2]oct-3-yl hydroxy(diphenyl)acetate; BZ) is a potent, atropine-like glycolic acid ester that blocks muscarinic receptors in the CNS and PNS (Spencer, 2000). During the 1950s, QNB was explored by both the United States and the Soviet Union as a potential antidote for organophosphorus anticholinesterases. However, the marked hallucinogenic actions of QNB led to its potential use as a CWA (Marshall, 1979). Saddam Hussein used QNB against the Kurds in July 1995 (Gaillard et al., 2014) and the Yugoslav People's Army allegedly used QNB leading to hallucinations and irrational behavior against Bosnian refugees fleeing from the town of Srebrenica (Hay, 1998; Gaillard et al., 2014).

QNB acts as a potent, competitive inhibitor of acetylcholine at muscarinic receptors located in the brain, heart, exocrine glands, and smooth muscles in various organs, resulting in a confused mental state with delusions, hallucinations, mental slowing, erratic behavior, a decrease in intestinal motility and tone, inhibition of bronchial and salivary secretions, and sweating, mydriasis, and tachycardia (Ketchum, 1963). Early studies demonstrated QNB was a potent and selective ligand for muscarinic receptors. Yamamura and Snyder (1974) showed that the inhibition of [^{3}H]-QNB binding to homogenates of rat brain by muscarinic drugs correlated with their pharmacological potencies, whereas nicotinic and noncholinergic drugs had negligible affinity. Kuhar and Yamamura (1976) reported that the injection of [^{3}H-]QNB into rat brain led to its localization to muscarinic receptors in the cerebral cortex, hippocampus, nucleus accumbens, and striatum. In addition, Jovic and Zupanc (1973) reported that the subcutaneous administration of QNB (5−20 mg/kg) to rats decreased oxygen consumption in the cerebral cortex and medulla oblongata. Interestingly, while QNB as a radioligand appears to bind to all known subtypes of muscarinic receptors, some evidence suggests that it has selectivity for the M2 subtype of muscarinic receptors when administered in vivo (McRee et al., 1995). QNB, thus, can produce a variety of neurological effects, presumably through the antagonism of cholinergic muscarinic receptor signaling in the CNS and PNS.

32.5 Concluding remarks and future directions

The nervous system has a number of biochemical, physiological, and anatomical characteristics that can make it particularly sensitive to CWAs. Indeed, many CWAs, in particular the nerve agents, can elicit debilitating and devastating neurotoxic responses. In some cases, these neurotoxic effects are elicited by direct interaction with specific target molecules within the nervous system, while in other cases actions on other organ systems can lead to indirect impairments of neurologic functions. The global importance of the continuous neuronal regulation of vital

physiological processes throughout the body makes the nervous system an effective target for disruption by CWAs. Thus, knowledge of mechanisms by which CWAs elicit neurotoxicity can aid in seeking and designing effective countermeasures against CWA exposures.

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Chapter 33

Behavioral toxicity of nerve agents

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33.1 Introduction

Behavioral changes in humans exposed to highly toxic organophosphorus (OP) compounds, called nerve agents, have been discussed in numerous reports. The incidence of behavioral effects is higher in individuals who have been severely exposed to nerve agents, but they may also occur in individuals who have received a low-level exposure below those producing convulsions and other severe clinical signs of toxicity. The behavioral effects usually start within a few hours and last from several days to several weeks or months. The most frequent symptoms include feelings of uneasiness, tenseness, and fatigue. Exposed individuals may be forgetful and generally display impaired memory and learning, poor comprehension, decreased ability to communicate, and occasional mild confusion.

There are a few reports describing behavioral changes in subjects accidentally exposed to nerve agents. They reported sleep disturbance, mood changes, fatigue, jitteriness or tenseness, an inability to read with comprehension, difficulties with thinking and expression, forgetfulness, a feeling of being mentally slowed, depression, irritability, giddiness, poor performance in arithmetic tests, minor difficulties in orientation, and frightening dreams. It was observed that the complex of central nervous system (CNS) symptoms may not fully develop until 24 h after exposure. In addition, no correlations between the presence or severity of symptoms and the degree of acetylcholinesterase inhibition were seen. Most of the effects of exposure disappear within 3 days. It was concluded that not only severe but also mild intoxication of nerve agents may cause behavioral and psychological disturbances. In general, the behavioral effects have not been permanent but have lasted from weeks to several months, or possibly several years. Long-term behavioral effects after poisoning with nerve agents or organophosphorus insecticides have been reported (Karczmar, 1984; Levin and Rodnitzki, 1976; Loh et al., 2010). These reports are based on clinical observations, which are occasionally supported by psychological studies.

33.2 The methods used to evaluate the behavioral effects of nerve agents

33.2.1 Functional observatory battery

The functional observatory battery is a noninvasive and relatively sensitive type of neurobehavioral examination of 40 sensory, motor, and automatic nervous functions. Some are scored (Table 33.1), and others are measured in absolute units (Frantik and Hornychova, 1995). The first evaluation is made when nerve agent-exposed or control rats are in the home cage. The observer evaluates each animal's posture, palpebral closure, and gait, and the presence or absence of convulsions is noted. Each rat is then removed from the home cage and briefly held by the observer. The presence or absence of spontaneous vocalization, piloerection, and other fur and skin abnormalities, as well as irritability, is noted. Lacrimation and salivation are also observed. Other signs such as exophthalmus, crustiness around the eyes, or emaciation are recorded too. The animals are then placed on a flat surface that serves as an open field. For a period of 3 min, the frequency of rearing responses is recorded. During the same time, gait characteristics are noted and ranked, and activity, tremor, convulsions, and abnormal posture are evaluated. At the end of the third minute, the number of fecal boluses and urine pools on the absorbent pad is registered. A reflex test that consists of recording each rat's response to the frontal approach of the blunt end of a pen, a touch of the pen to the posterior flank, and an auditory click stimulus is then used. The responsiveness to a pinch on

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TABLE 33.1 Functional observational battery.

Marker	Scored values only									
	−2	−1	0	1	2	3	4	5	6	7
Posture				Sitting or standing	*Rearing*	*Asleep*	Flattened	Lying on side	Crouched over	Head bobbing
Catch difficulty				Passive	*Normal*	Defense	Flight	Escape	Aggression	
Ease of handling				Very easy	Easy	Moderately difficult	Difficult			
Muscular tonus	Atonia	Hypotonia	*Normal*	Hypertonia	Rigidity	Fasciculations				
Lacrimation			*None*	Slight	Severe	Crusta	Colored crusta			
Palpebral closure				*Open*	Slightly	Half-way	Completely shut	Ptosis		
Endoexophthalmus		Endo	*Normal*	Exo						
Piloerection			*No*	Yes						
Skin abnormalities			*Normal*	Pale	Erythema	Cyanosis	Pigmented	Cold	Injury	
Salivation			*None*	Slight	Severe					
Nose secretion			*None*	Slight	Severe	Colored				
Clonic movements			*Normal*	Repetitive	Nonrhythmic	Mild tremors	Severe tremors	Myoclonic	Clonic	
Tonic movements			*Normal*	Contraction of extensors	Opisthotonus	Emprosthotonus	Explosive jumps	Tonic convulsions		
Gait			*Normal*	Ataxia	Overcompensation of hindlimb movement	Feet point outwards from body	Forelimbs are extended	Walks on tiptoes	Hunched body	Body is flattened against surface
Gait score				*Normal*	Slightly impaired	Somewhat impaired	Totally impaired			
Mobility score				*Normal*	Slightly impaired	Somewhat impaired	Totally impaired			
Arousal (level of unprovoked activity)				Very low	Sporadic	Reduced	*Normal*	Enhanced	Permanent	
Tension			*None*	Partial (ears)	Stupor					

(*Continued*)

Stereotypy			*None*	Head weaving	Body weaving	Grooming	Circling	Others		
Bizarre behavior			*None*	Head	Body	Self-mutilation	Abnormal movements	Others		
Approach response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Touch response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Click response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Tail-pinch response				No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction		
Pupil size		Miosis	*Normal*	Mydriasis						
Pupil response			No reaction	*Normal reaction*						
Righting reflex				*Normal*	Slightly uncoordinated	Lands on side	Lands on back			

the tail and the ability of the pupils to constrict in response to light are then assessed. These measurements are followed by a test for the aerial righting reflex, then by the measurements of forelimb and hindlimb grip strength, body weight, rectal temperature, and finally hindlimb landing foot splay. This entire battery of tests requires approximately 6–8 min per animal. Motor activity data are collected using an apparatus for testing a spontaneous motor activity of laboratory animals. The animals are placed in the measuring cage for a period of 10 min and their movements (total horizontal activity, stereotypical activity, rearing, jumping, scratching, and total vertical activity) are recorded.

33.2.2 Performance on the RAM task

RAM sessions are conducted using an eight-arm commercially available radial maze measuring 137.2 cm in diameter. The center of the maze is a plastic octagon hub measuring 26.67 cm across, with a Plexiglass lid and wire grid floor. A Plexiglass arm with a wire mesh floor is attached to each of the eight sides of the hub. The entrance to each arm contains a motorized guillotine door allowing access to and from the hub. Each arm's runway contains two floor-mounted switches, which are depressed by the weight of the rat when present in the proximal and distal portion of the runway, respectively. The terminal portion of each arm contains a food dispenser, for delivering food pellets, connected to a trough that is outfitted with the photoemitter/detector that can detect access by the rats. Experiments are controlled and monitored using a commercial hardware interface and a microcomputer using the L2T2S software control system (Coulbourn Instruments). For the RAM task, four of eight arms are "baited," meaning that a single food pellet is available upon a nose-poke into the food trough at the terminal portion of each arm. Each rat is randomly assigned a maze configuration of four baited arms from 37 possible configurations, excluding more than two consecutive baited arms. The same configuration of baited arms is used for a specific rat for each of the sessions, but different configurations can be used for different rats. Sessions begin with the rat placed in the center hub compartment and the doors to the eight arms are raised. The rat is then free to explore the maze to obtain the food rewards available from the four baited arms. The session is terminated when the rat obtains all four food rewards or when 15 min have elapsed. If a rat does not complete the maze within 15 min, a completion time of 15 min is assigned and errors are analyzed. Failure to complete the maze, however, is infrequent and only occurs during the initial few sessions in the maze. No familiarity training with the maze is conducted prior to the first session. The major dependent variables characterizing performance on the RAM task are the time to complete the maze and the number of errors made. Errors are designated when a rat chooses an unbaited arm (reference memory error) or when a rat returns to a baited arm after obtaining the food reward (working memory error) (Genovese et al., 2006).

33.2.3 Acoustic startle response and prepulse inhibition

The animals are tested for acoustic startle response and prepulse inhibition in the SM100 Startle Monitor system. The system is programmed for six types of white-noise burst stimulus trials: no stimulus (background, 60 dB), prepulse (70 dB), pulse (100 and 120 dB), and prepulse plus pulse (70 dB + 100 dB and 70 dB + 120 dB). Each trial type is presented 10 times in 10 blocks. Stimuli are presented in random order to avoid order effects and habituation. The intertrial interval can vary from 9 to 16 s. All animals are regularly handled before individual tests in order to minimize handling-related stress. Animals are pair matched according to baseline values into experimental groups using the average of the response to 100 and 120 dB. The tested animals are restrained loosely in holders that are placed on a sensing plate transforming movements of the body (jerks) into an analog signal through an interface. Finally, the percentage of prepulse inhibition measurements is calculated as the difference between the pulse alone, multiplied by 100. Percentage scores are typically used to minimize the effect of individual variation of startle amplitude on prepulse inhibition (Mach et al., 2008).

33.2.4 Performance on the Y-maze

Cognitive functioning can be tested using a Y-maze with averse motivation by a strong electric footshock, evaluating learning and spatial memory (Koupilova et al., 1995). The Y-maze is a plastic box consisting of a square start area (285 × 480 mm) separated by a Plexiglass sliding door from two trapezoid, black and white arms—the choice area (140 × 324 mm). The grid floor in the start and choice area is electrifiable. The animal is placed on the start area and after 48 s electric footshocks (60 V, 50 Hz, duration 0.5 s) are applied at 5-s intervals. The rats try to avoid the shock by escaping to one of two arms. In the case of a rat moving to the wrong (dark) arm, the rat fails to avoid further footshock. The animals are taught spatial discrimination with the preference of the black or white arm in the Y-maze. The latency to enter the correct arm is measured and the number of wrong entries is counted. Before exposure to a nerve agent, the rats are trained to avoid footshock by moving to the correct (white) arm in the Y-maze. It usually takes 4 weeks of training to reach the criterion of 80% or more correct

averse behaviors (moving to the correct arm) within less than 1.5 s. During the training, 10 sessions (two trials/session) per week lasting 4 min each are realized. The exposure starts the day after the animals reach this criterion. The latency time to enter the correct arm by nerve agent-exposed rats and the number of entry errors are compared to the values obtained from the control rats exposed to pure air instead of a nerve agent.

33.2.5 Performance on the T-maze

Cognitive functioning can be also tested using a T-maze, consisting of five segments, a starting and a goal compartment to evaluate learning, spatial memory, and spatial orientation (Koupilova and Herink, 1995). The rats are trained with a food reward, to run through the maze in less than 10 s without entering the side arm. The time necessary to reach the goal box is recorded. Before exposure to a nerve agent, the rats are trained to reach the goal box as soon as possible by moving to the correct segment in the T-maze. It usually takes 4−6 weeks of training to reach the criterion of 80% or more correct behavior. The exposure starts the day after the animals reached this criterion. The time of reaching the goal box by nerve agent-exposed rats is compared to the values obtained from the same rats immediately before nerve agent exposure and from control rats exposed to pure air instead of a nerve agent.

33.2.6 Performance on the Morris water maze

The water maze (WM) is often used for the evaluation of effects of various compounds on memory functions, that is, memory formation, consolidation, and retrieval effects due to its advantages and broad utilization. The Morris WM is a widely used measurement of visuospatial learning that has been demonstrated to have high validity in identifying the cognitive effects of various brain lesions and the effects of drugs used to treat cognitive deficits (Morris, 1984; Myhrer, 2003). Special motivation such as food and water deprivation is not required for the WM performance. The effect of odor cue is eliminated in the WM. In addition, rats are forced to swim in the WM. They cannot choose whether or not to move, so failure to respond is not a confound (Shukitt-Hale et al., 2004). The place learning version with a submerged platform can be used for working memory tests (Myhrer, 2003). The WM can be used to measure spatial learning and memory in the case of the evaluation of cognitive impairment in rats because of the mentioned advantages. In addition, a visuospatial learning task is sensitive to cholinergic dysfunction and, thus, deficits in visuospatial processing have been recognized as some of the negative consequences in organisms chronically exposed to OPC (Roldán-Tapie et al., 2005).

The rats perform cognitive tasks that require spatial learning and memory—the ability to acquire a cognitive representation of location in space and the ability to effectively navigate the environment in the WM (Shukitt-Hale et al., 2004). Memory alterations appear to occur mostly in secondary memory systems and are reflected in the storage of newly acquired information (Bartus et al., 1989; Joseph, 1992). It is thought that the hippocampus mediates allocentric spatial navigation (i.e., place learning) and that the prefrontal cortex is critical to acquiring the rules that govern performance in particular tasks (i.e., procedural knowledge), while the dorsomedial striatum mediates egocentric spatial orientation (i.e., response and cue learning) (McDonald and White, 1994; Oliviera et al., 1997).

The WM consists of a black circular pool (180 cm diameter × 80 cm high) filled to a depth of 25 cm with room temperature water (Raveh et al., 2002). The pool is imaginarily divided into four compartments numbered clockwise 1−4. The black antireflective circular escape platform (15 cm diameter) is placed into compartment 1 or 4, 20 cm off the pool wall. The platform is sunk 2 cm below the water surface, so that it is not visible from to the rat via the water mirror effect. A yellow rectangle (30 cm × 40 cm) is fixed on the pool wall, immediately close to the platform, as the spatial conditional cue (Robinson et al., 2004). Its place is variable according to the platform. Another dark rectangle is randomly fixed on the pool wall in different compartments (without platform) as the negative conditional cue. Around the pool, there are several stable extramaze cues in the room that the rat could use to navigate the maze (Morris, 1984). However, the impact of extramaze cues is not significant due to high maze walls.

33.2.7 Performance on the passive avoidance test

Passive avoidance is a fear-motivated test classically used to assess short-term or long-term memory on small laboratory animals (rats, mice). Basically, passive avoidance working protocols involve timing of transitions, that is, time that the animal takes to move from the white compartment to the black one after a conditioning session in which entry into the black compartment is punished with a mild inescapable electrical shock. The animal's position is detected using high-sensitivity weight transducers providing more effective and reliable detection of animal responses (zones entries) than systems based on photocell beams or on grid floor displacements. The measured parameter is a latency to enter the black compartment.

A two-compartment step-through passive avoidance apparatus is usually used. The apparatus (430 × 220 × 190 mm) is divided into bright and dark compartments by a removable divider. The divider is a tunnel with an automatic door. Using a software-controlled magnetic switch, this door can be closed automatically. The gate has to be opened manually at the start of the experiment. Each compartment is equipped with a light (2.2 W). The ceiling also contains a common loudspeaker to deliver a sound signal (10 kHz fixed, max. 100 dB). The animal's position inside the cage is monitored with the help of infrared light barriers. After a specified time interval following drug administration, passive avoidance training is performed. The animals are placed in the bright compartment and allowed to explore for 30 s, at which point the guillotine door is raised to allow the rat to enter the dark compartment. When the animal enters the dark compartment, the guillotine door is closed and an electrical footshock (0.1−3.0 mA) is delivered. Training sessions are conducted twice (double-trainings) during the light phase of the 12-h day/night cycle. The second double-training session is carried out immediately after the first. The animals are placed in the bright compartment and allowed to explore for 30 s, and then the guillotine door will raise. The latency to enter the dark compartment is recorded for up to 300 s (Gacar et al., 2011).

33.2.8 Performance on the Barnes maze

The Barnes maze tests spatial learning and memory (Pompl et al., 1999). It consists of a platform (91 cm in diameter) that is elevated 91 cm from the ground with 20 holes (5 cm in diameter) equally spaced around the outer perimeter of the platform to include one "escape hole" under which is a small escape box. Posters with black and white geometric shapes are used as spatial cues and placed on the walls surrounding the maze (Sharma et al., 2010). Two lights and a camera are positioned above the apparatus to provide light and record the animals' movements, respectively. After a 10-min acclimatization period, the animals are transported to the maze in an opaque cup and placed in the center of apparatus. After 30 s, the cup is lifted and a 5-min trial begins. Each animal is placed in the maze for four trials with 15−25 min between trials (Sharma et al., 2010). Latency to escape (a measure of spatial learning and memory), the number of head pokes into the perimeter holes (a measure of error), and the primary latency to head poke into the escape hole (Sharma et al., 2010; Patel et al., 2014) are recorded using ANY-maze software. If the animal successfully escaped (defined as staying in the escape hole for 15 s), the test ended and the animal remained in the hole, covered by the experimenter, for 30 s to reduce anxiety (Fox et al., 1998). If the animal did not successfully locate the escape hole within the testing period, it is picked up and placed in the escape hole, where it remains for 1 min (Fox et al., 1998; Cheng et al., 2014).

33.3 Long-term behavioral effects of acute high-level exposure to nerve agents

Many of the data regarding long-term neurological sequelae to exposures to cholinesterase inhibitors in humans have been gathered following accidental exposures to OP compounds (pesticides as well as nerve agents). Nevertheless, the extrapolation from these exposures to prediction of effects from nerve agents is difficult for several reasons:

- The cholinergic crisis caused by acute, severe intoxication with OP pesticides is generally much longer than that caused by OP nerve agents;
- OP pesticide-induced delayed peripheral neuropathy can be caused by nerve agents only at doses many times greater than the LD_{50} (Davis et al., 1960);
- A delayed manifestation of OP poisoning has not been described after administration of nerve agents to animals or in instances of nerve agent poisoning in humans (Sidell, 1997).

There have been descriptions of the acute effects in humans that follow high-dose exposure ($\geq LD_{50}$) to the nerve agents soman, sarin, and VX (Inoue, 1995; Nakajima et al., 1997; Nozaki et al., 1995). A similar cluster of behavioral symptoms (anxiety, psychomotor depression, intellectual impairment, and sleep disturbance) was observed in the immediate period following resolution of the acute signs of intoxication that then slowly faded with time, sometimes taking months to be fully resolved. The CNS symptoms noted following short-term exposure of humans to diisopropyl fluorophosphate (DFP) were excessive dreaming, insomnia, jitteriness and restlessness, increased tension, emotional lability, subjective tremulousness, nightmares, giddiness, drowsiness, and mental confusion. CNS symptoms were correlated with the depression of red blood cell acetylcholinesterase (AChE; EC 3.1.1.7) to 70% − 60% of original activity and they disappeared within 1−4 days (Grob et al., 1947). It was also noted that more severely exposed individuals and those with multiple exposures tended to display persistent symptoms that included forgetfulness, irritability, and confused thinking, although the duration of these persistent symptoms was never clearly defined (Holmes and Gaon, 1956). These CNS symptoms are virtually identical to those that have been reported to occur following high-level exposure to nerve agents. It was shown in the study of human sarin poisoning that sarin-induced behavioral effects were virtually identical to those reported for DFP.

These effects coincided with the depression of plasma ChE and red blood cell AChE activity to approximately 60% and 50%, respectively, of original activity (Grob and Harvey, 1958). The behavioral symptoms such as anxiety, psychomotor depression, a general intellectual impairment consisting of difficulties in concentration and retention, and sleep impairment generally involving insomnia due to excessive dreaming were also described during human poisoning with the nerve agent VX (Bowers et al., 1976).

Exposure to high doses of OP compounds including nerve agents has been demonstrated to result in severe brain neuropathology that involves not only neuronal degeneration and necrosis of various brain regions (Lemercier et al., 1983; McLeod et al., 1982; Petras, 1981) but also persistent severe alteration in behavior and cognitive incapacitation, especially impairments of learning and memory (Bushnell et al., 1991; McDonald et al., 1988). The most significant injury caused by OP poisoning is neuronal degeneration of the hippocampus that is associated with spatial learning and memory. Therefore, impairment of cognitive functions, especially incapacitation of learning and memory, belongs to the most frequent central signs of acute OP poisoning (Marrs, 1993; McDonald et al., 1988). In addition, the adverse effects of OP compounds on cognition functions, such as learning and memory, may persist for quite some time after termination of toxicant exposure. The results from several studies have demonstrated the presence of OP compound-induced learning impairments several days after the classic signs of OP toxicity have subsided (Buccafusco et al., 1990; Bushnell et al., 1991; McDonald et al., 1988). Behavioral effects are typically evident before the occurrence of physical symptoms. These effects were associated with whole-blood ChE inhibitions of $>60\%$. It was also found that severe poisoning with OP compounds causing general tonic-clonic convulsions altered Ca^{2+} dynamics that could underlie some of the long-term plasticity changes associated with OP compound toxicity. These changes demonstrated in hippocampal neurons can be responsible for the long-term behavioral toxicity of OP compounds (Deshpande et al., 2010, 2016).

Several studies of the long-term effects of sarin exposure on victims from Japan have been published. Exposure to nerve agents in humans was found to produce effects that include cognitive deficits and memory loss (Hatta et al., 1996; Hood, 2001; Okudera, 2002). Eighteen victims of the Tokyo subway incident were evaluated at 6–8 months postexposure (Yokoyama et al., 1998). Sarin-exposed individuals scored significantly lower than controls on a digit symbol substitution test, and scored significantly higher than controls on a general health questionnaire (GHQ, psychiatric symptoms) and a profile of mood states (POMS, fatigue). The elevated scores on the GHQ and POMS were positively related to increased PTSD (posttraumatic stress disorder) scores and were considered to be due to PTSD (Yokoyama et al., 1998). There have been two brief reports of severely poisoned nerve agent victims (one sarin and one VX) in Japan who experienced retrograde amnesia, possibly due to prolonged periods of seizures and/or hypoxia (Hatta et al., 1996; Nozaki et al., 1995). Symptoms related to sarin exposure in Japan still existed 1–3 years after the incident and included fatigue, asthenia, shoulder stiffness, and blurred vision (Abu-Qare and Abou-Donia, 2002).

The existence of long-term behavioral effects following acute exposure to high doses of nerve agents and OP pesticides was verified many times with the help of laboratory experiments on animals. There are numerous studies in animals showing that survivors of high-level OP exposure can experience subtle but significant long-term neurological and neuropsychological outcomes that are detectable months or even years following recovery from acute poisoning (Brown and Kelley, 1998). Three months after severe paraoxon exposure, the surviving rats exhibited a despair-like state, anhedonia-like condition, increased anxiety, and impaired recognition memory (Deshpande et al., 2014). Exposure of animals to nerve agents was shown to produce neurotoxicity in the CNS areas associated with cognition and memory functions (Koplovitz et al., 1992; Petras, 1994). There are a few studies that have revealed changes in the brain following sublethal nerve agent exposure that involve not only the cholinergic system but also the glutamatergic system (Lallement et al., 1992; McDonough and Shih, 1997). Excitotoxic injury caused by increased levels of glutamate has repeatedly been shown to cause cognitive dysfunction (O'Dell et al., 2000). Therefore, the disruption of cognitive functions, especially spatial and working memory, seems to be the most frequent and most observable behavioral effect of nerve agent poisoning. In addition, acute and repeated administration of OP compounds induced anxiogenic and depression-like responses. This fact was assessed on the elevated plus-maze and forced-swim tests, which are validated animal models to observe for anxiety and depression-like behavior (Assissi et al., 2005). These studies show changes in the brain following sublethal nerve agent exposure that lead to memory and attention deficits that normally involve the hippocampus (Hatta et al., 1996; Miyaki et al., 2005; Nishikawi et al., 2001). The role of the hippocampus in complex visuospatial learning and memory has been well established. The high concentration of NMDA and AMPA glutamate receptors, which play a key role in hippocampal-mediated learning and memory, also make the hippocampus highly vulnerable to glutamate-induced excitotoxic injury from nerve agent poisoning (Filliat et al., 2007; Lallement et al., 1992; Shih et al., 1990).

Following a high-dose exposure (above 0.5 LD_{50}), seizures are a prominent sign of nerve agent intoxication and these prolonged seizures can produce neural lesions

(McDonough and Shih, 1997). Thus, neurological and behavioral deficits are predictable long-term effects following exposure to such doses of nerve agents. Animals exposed to high (convulsive) doses of nerve agent can develop spontaneous seizures, and display hyperactive and aggressive behavior and profound deficits in learning and/or performance of a variety of behavioral tasks. Animal studies have demonstrated deficits in acquisition of several types of operant tasks, performance of serial probe recognition task, maze learning, and passive avoidance learning following acute poisoning with nerve agents (McDonough et al., 1986; Modrow and Jaax, 1989; Raffaele et al., 1987).

The inhalation exposure to high-level sarin-induced impaired memory processes in rats seen at 1 month postexposure with no recovery of cognitive function during the 6-month follow-up period. In the open field, sarin-exposed rats showed a significant increase in overall activity with no habituation over days. In a working memory paradigm in the WM, the same rats showed impaired working and reference memory processes with no recovery. These data suggest long-lasting impairment of brain functions in surviving rats following a single sarin exposure. Animals that seem to fully recover from the exposure, and even animals that initially show no toxicity signs, develop some adverse neurobehavioral changes with time (Grauer et al., 2008). These findings are in accord with reports on long-term behavioral impairment following exposure to OP pesticides used in agriculture (Wesseling et al., 2002). Similarly, long-term follow-up of victims of the sarin attacks in Japan demonstrated neurological as well as emotional and cognitive changes up to 7 years postexposure (Miyaki et al., 2005; Ohbu et al., 1997; Yokoyama et al., 1998).

Generally, according to high-dose exposure studies, animals exposed to nerve agents that exhibit seizures that are not promptly controlled develop brain damage and subsequent neurobehavioral problems. Animals that do not develop seizures or those that are rapidly and effectively treated with drugs that stop the seizures suffer no brain lesions and display no long-term neurobehavioral deficits. Besides classic antidotes (atropine, oximes, benzodiazepines), other drugs, such as the centrally acting anticholinergic drug scopolamine and a new anticonvulsant drug imidazenil, are effective in eliminating or at least reducing behavioral toxicity of nerve agents (Che et al., 2011; Wang et al., 2012).

33.4 Chronic behavioral effects of single or repeated low-level exposure to nerve agents

Anticholinesterase compounds such as nerve agents can alter behavioral functions even after small subtoxic doses. There are very few data on human exposures. Based on the data describing the signs and symptoms in accidentally exposed humans, some long-term health effects, including behavioral effects of repeated subclinical exposures to OP compounds, were observed (Wesseling et al., 2002). When workers were exposed to small amounts of nerve agents, they showed mild toxic signs of exposure including CNS effects such as insomnia, excessive dreaming, restlessness, drowsiness, and weakness (Craig and Freeman, 1953). It was shown that psychological symptoms are probably more common than usually recognized and may persist in more subtle forms for much longer (days, weeks) than physical symptoms (Sidell and Hurst, 1997). Recently, a dose−response association was found between low-dose exposure to sarin and cyclosarin inhalation during the 1991 Gulf War and impaired neurobehavioral functioning as well as subtle CNS pathology as revealed by MRI study (Heaton et al., 2007; Proctor et al., 2006). It is interesting that functional impairments were detected even in people who initially developed only mild or no signs of sarin or cyclosarin toxicity. These data correspond to the published epidemiological studies showing alterations in cognitive functions, impaired memory, and concentrations in humans after chronic low-dosage occupational exposure to OP insecticides (Parrón et al., 1996; Stephens et al., 1995). An increased amount of forgetfulness and difficulties in thinking, exposure-related increases in work-related tension, sleep disturbance, restlessness, and nervousness have been documented among sheep farmers exposed to OP pesticides (Beach et al., 1996; Stephens et al., 1995). Recently published meta-analysis of 14 studies which fulfilled strict criteria for inclusion and data from more than 1600 participants, found an association between subthreshold OP exposures and impaired neurobehavioral functions. The domains of cognition most affected included attention, working memory, executive function, visuospatial ability, and visual memory (Ross et al., 2013).

Based on experimental animal data, the progression of signs, their neuropharmacological basis, and the toxic consequence elicited from acute high-dose exposures have been well characterized (McDonough and Shih, 1993; Shih et al., 2003). However, much less is known about the long-term effects of repeated low-dose nerve agent exposure. Several comprehensive reviews on the long-term health effects of low-level nerve agent exposure have been published (Moore, 1998; Romano et al., 2001). There are prospective animal studies that support the argument that chronic or repeated exposures to OPs at levels that are not associated with acute toxicity can indeed result in a variety of sustained deficits in delayed matching performance, sensorimotor gating, spatial learning and retention, recognition memory, and cognitive flexibility. They have been reported in association with

low-level exposures to OPs (Terry et al., 2012; Yan et al., 2012).

It is known that a significant, clinically manifested AChE inhibition in the CNS leading to the neuronal degeneration of some brain regions including the hippocampus, associated with spatial learning and memory, is not necessary for clinically manifested cognitive impairments. This fact corresponds with earlier published data about neurological and neurophysiological outcomes detectable months or even years following recovery from acute OP poisoning (Savage et al., 1988; Yokoyama et al., 1998). It is very difficult to find the real reason for the memory impairments in the case of low-level nerve agent exposure. Recently, a temporal relationship has been demonstrated between OP-induced impairment in performance of a spatial memory task and the protracted decrease in the expression of cholinergic receptors in specific brain regions (including the hippocampus) following the asymptomatic exposure to OP compounds (Stone et al., 2000). Nerve agent-induced impairment of cognitive functions is probably caused by subsequent desensitization and internalization of cholinergic receptors as a reaction of nerve agent-exposed organisms on hyperstimulation of cholinergic receptors, especially in parts of the brain with a high density of cholinergic synapses, such as the hippocampus (McDonald et al., 1988; Stone et al., 2000). This means that a decrease in the number of cholinergic receptors in the hippocampus following low-level exposure to OPs without significant AChE inhibition could cause memory impairments.

However, noncholinergic pathways may also be involved in OP-induced alteration of behavioral functions. The above-mentioned prospective studies support the argument that chronic or repeated exposures to OPs at levels that are not associated with acute toxicity can indeed result in cognitive deficits. The neurobiological substrates of these persistent behavioral effects of OPs have not been fully elucidated but substantial evidence now suggests that cholinesterase inhibition alone is insufficient as an explanation. It has been suggested that interactions of OPs with noncholinesterase targets may contribute to the more delayed and persistent effects observed following chronic or repeated exposures (Costa, 2006). The list of noncholinesterase targets for OPs is growing and now includes a variety of proteins, receptors, and enzymes (Casida and Quistad, 2005; Terry, 2012). There are other serine hydrolases that could be the secondary targets of these agents (Casida and Quistad, 2005; Rocha et al., 1999). Studies carried out in AChE knockout mice have also shown that some OP-induced effects, including behavioral effects, are independent of their cholinergic effects (Duysen et al., 2001). It is supposed that low levels of OP compounds induce changes in the brain dopaminergic and serotoninergic systems and that these changes may help to explain the various neuropsychological symptoms associated with low-level OP exposure. It was shown that the nerve agent sarin can induce significant long-term changes on the monoamine activity in different brain structures of mice. These effects of sarin on the monoaminergic neurotransmitter systems suggest that OPs exert some of their long-lasting toxic effects including behavioral effects via noncholinergic mechanisms (Oswal et al., 2013). OP compound-induced impairment of cognitive functions may involve multiple mechanisms of action which may be due to deficits in axonal transport, mitochondria, and neurotropic factors (Terry, 2012). Many results indicate that the threshold for neurotoxic consequences may be surpassed during repeated exposure to subthreshold doses of OPs (Singh et al., 2016; Terry et al., 2014). It was described that OP compound-induced cognitive deficits may be related to persistent functional changes in brain neurotrophin and cholinergic pathways (Terry et al., 2011). Additionally, the influence of diet on the behavioral toxicity of nerve agents was demonstrated (Langston and Myers, 2011). It was found that diet composition exacerbates or attenuates the behavioral toxicity of nerve agents in exposed rodents. The exacerbated behavioral toxicity of nerve agents in animals fed a glucose-enriched diet could be due to inactivation of esterases including AChE, BuChE, CAE, and PON by glycation or glyco-oxidation or by increased acetylcholine synthesis and/or its utilization (Myers and Langston, 2011). On the other hand, a ketogenic diet attenuates behavioral toxicity of nerve agents. The mechanism responsible for this effect is unknown but there is considerable evidence that the ketogenic diet has neuroprotectant and anticonvulsant properties by altered energy or altered neurotransmitter (GABA) synthesis (Bough and Rho, 2007). In addition, a moderate benefit of choline supplementation against behavioral toxicity of nerve agents was observed (Langston and Myers, 2011). Moreover, the influence of genetic background on the toxicity of nerve agents was also demonstrated (Matson et al., 2018a). The available data suggest genetically determined differences in susceptibility or resistance to nerve agent-induced toxicity, including behavioral toxicity. Some genetic markers for organophosphorus susceptibility (variants of the gene paraoxonase 1, cytochrome gene variants, butyrylcholinesterase gene variants) in humans have also been identified and confirmed in rodent models (Furlong, 2007; Povey et al., 2007; Lockridge, 2015). Thus, genetic background plays an important role in behavioral, neurological, and physiological responses to nerve agent exposure. This fact was supposed by the assessment of mouse strain differences in toxic response to sarin. It was found that BALB/cByJ and FVB/NJ strains were the most resistant strains, while DBA/2J was the most sensitive mouse strain (Matson et al., 2018b).

In the literature available on repeated low-dose exposure to nerve agents, soman is the nerve agent studied most often. Mice, rats, guinea pigs, and primates have been used to investigate repeated low-dose soman exposure. The effects of repeated soman exposures ranged from performance decrements on a well-learned compensatory tracking task (Blick et al., 1994b) to development of attention deficits (Gause et al., 1985) and hyperreactive responses to handling (Shih et al., 1990). In addition, a single subcutaneous exposure of guinea pigs to sublethal doses of soman triggered long-lasting anxiogenesis and decreased locomotor activity (Mamczarz et al., 2010).

Unlike soman, the amount of literature regarding the effects of repeated low-level exposure to sarin is rather sparse and sometimes conflicting. Rhesus monkeys exposed to low levels of intramuscular sarin showed no signs of adverse health or long-term behavioral effects (Burchfiel et al., 1976). In contrast, it has been observed in rats and mice that intraperitoneal injections of subtoxic doses of sarin or soman decreased locomotor activity and altered behavior on the plus-maze and elevated horizontal bridge tests (Baille et al., 2001; Nieminen et al., 1990; Sirkka et al., 1990). Single as well as repeated low-level inhalation exposure of rats to sarin produced a deficit on the RAM spatial memory task. The deficit was resolved during the first 3 weeks of acquisition (Genovese et al., 2009). It was also shown that repeated low-level inhalation of sarin in rats at clinically asymptomatic doses was disruptive to neurophysiological function and caused long-term memory impairments (Kassa et al., 2001a,b). The results of the study related to the measurement of sarin-induced alteration of behavioral and neurophysiological functions at 3 months following low-level sarin inhalation exposure of rats showed a significant alteration of mobile activity and gait characterized by ataxia and an increase in stereotypical behavior. These signs were observed in rats repeatedly exposed to sarin at clinically asymptomatic doses or singly exposed to sarin at doses causing mild muscarinic signs of exposure. These animals had awkward hindlimbs and their mobility was markedly diminished (Kassa et al., 2001d). Spatial discrimination in the Y-maze was also altered in rats exposed to low levels of sarin. While spatial orientation of rats singly exposed to clinically asymptomatic doses of sarin was significantly influenced for a short time only (1 or 2 h following exposure), the rats repeatedly exposed to clinically asymptomatic doses of sarin showed a decrease in Y-maze performance for a relatively long time (until the third week following the exposure) (Kassa et al., 2004). The significant impairment of spatial memory of rats exposed to clinically asymptomatic concentrations of sarin was also observed when cognitive functions were evaluated with the help of T-maze performance. Rats exposed to low-level sarin showed a significant decrease in T-maze performance for a short time (until the first day following the exposure). In addition, the effects of low-level sarin inhalation exposure were dose dependent. When the rats were exposed to low-level sarin causing moderate signs of poisoning, their time of passage through the maze was more lengthened at 1 and 2 h following the inhalation exposure compared to the rats exposed to clinically asymptomatic levels of sarin (Kassa et al., 2001c).

A single exposure to another nerve agent, cyclosarin, at concentrations that do not produce convulsions or severe clinical signs of toxicity can also produce performance deficits on learned behavioral tasks. However, with repeated exposure, the deficits are not persistent and recovery is complete. In addition, exposure concentrations not producing any evaluated clinical signs of toxicity, other than temporary miosis (in the case of inhalation exposure), do not produce performance deficits on behavioral tasks (Genovese et al., 2006). Asymptomatic exposure of rats to VX vapors can produce only minor performance effects on previously learned behavioral tasks and on the acquisition of a new behavioral task (Genovese et al., 2007).

Reports in the literature of animal studies show that nerve agents can be administered repeatedly with minimal overt neurobehavioral effects if care is taken in choosing the dose and time between doses (Sterri et al., 1980, 1981). Repeated low-level nerve agent exposure made the cognitive impairments longer and higher compared to single nerve agent exposure. Repeated exposure to low doses of soman can produce small, transient performance decrements only, most likely due to the development of a physiological and behavioral tolerance to low levels of ChE activity (Blick et al., 1994a,b). Nevertheless, a progressive and long-lasting inhibition of AChE in the CNS following repeated administration of low doses of soman was demonstrated (Hartgraves and Murphy, 1992). This study was corroborated by Olson et al. (2000) using sarin. Generally, repeated or long-term exposure to low levels of nerve agents can cause neurophysiological and behavioral alterations (Abu-Qare and Abou-Donia, 2002).

Rats repeatedly exposed to sarin at doses corresponding to $0.5 \times LD_{50}$ (three times per week, s.c.) showed an increase in acoustic startle and a decrease in distance explored in the open field 2 weeks after sarin exposure. On the other hand, no effect of sarin exposure on passive avoidance was noted at the same time after sarin poisoning. Brain regional AChE was not affected at any time after sarin exposure, but muscarinic receptors were downregulated in the hippocampus, caudate putamen, and mesencephalon in the sarin group at 2 weeks postexposure.

Thus, downregulation of muscarinic receptors in the hippocampus as a reaction to acetylcholine accumulation at muscarinic receptor sites based on AChE inhibition can be considered a cause of behavior performance deficits, especially disruption of cognitive functions (Scremin et al., 2003). In addition, protracted impairment of cognitive functions in rats exposed repeatedly to low-level OP compounds may be associated with a decreased rate of AChE recovery in the hippocampus (Prendergast et al., 1997).

The results from several studies have demonstrated the presence of OP-induced learning impairments several days after the behavioral signs of OP toxicity have subsided (Bushnell et al., 1991; McDonald et al., 1988). Chronic exposure to OP compounds can also result in specific long-term cognitive deficits even when signs and symptoms of excessive cholinergic activity are not present (Prendergast et al., 1998). Thus, the significant, clinically manifested AChE inhibition in the CNS leading to the neuronal degeneration of some brain regions, including the hippocampus, is not necessary for the clinically manifested cognitive impairments. This conclusion corresponds with earlier published data about neurological and neurophysiological outcomes detectable months or even years following recovery from acute OP poisoning (Savage et al., 1988; Yokoyama et al., 1998). A current study attempts to show a temporal relationship between OP-induced impairment in performance of a spatial memory task and the protracted decrease in the expression of cholinergic receptors in specific brain regions caused by asymptomatic exposure to an OP compound (Stone et al., 2000). In addition, low-level OP-induced memory impairment may be associated with a decreased AChE recovery in the hippocampus relative to the cortex. This decreased rate of enzyme recovery may contribute to hippocampal toxicity underlying protracted impairment of working memory and other cognitive functions (Prendergast et al., 1997).

Repeated or chronic low-level nerve agent exposure can cause a prolonged inhibition of extracellular AChE leading to a prolonged increase in extracellular acetylcholine (ACh). The prolonged availability of ACh in the synaptic clefts results in feedback inhibition on muscarinic, presynaptic receptors to decrease further ACh release (Russell et al., 1985). The greater ACh release in the nerve agent-exposed group may be due to the known downregulation of muscarinic receptors in response to chronic nerve agent exposure (Churchill et al., 1984). Neurochemical analyses showed that normal brain neurotransmitter and receptor homeostasis is disrupted even at 10–12 days after 2 weeks of chronic nerve agent exposure at least in the striatum but probably throughout the whole cholinergic system in the brain (Shih et al., 2006).

33.5 Concluding remarks and future directions

Exposure to high doses of nerve agents has been demonstrated to result in severe brain neuropathology that involves not only neuronal degeneration and necrosis of various brain regions, but also persistent severe alterations in behavior and cognitive functions, especially impairment of learning and memory. The most significant injury caused by nerve agent poisoning is neuronal degeneration of the hippocampus, which is associated with spatial learning and memory. Therefore, impairment of cognitive functions, especially incapacitation of learning and memory, belongs to the most frequent central signs of acute nerve agent poisoning. In addition, the adverse effects of nerve agents on cognitive functions, such as learning and memory, may persist for a relatively long time following the termination of nerve agent exposure.

Behavioral alterations and impairments of cognitive functions were found following acute exposure to nerve agents with the absence of any classic signs of cholinergic toxicity. It was shown based on the experimental results that not only convulsive doses but also clinically asymptomatic doses of nerve agents can cause subtle long-term neurophysiological and neurobehavioral dysfunctions. The neurological and neurophysiological outcomes are detectable months or even years following the recovery from acute poisoning. It probably means that systems other than the cholinergic nervous system can be involved in nerve agent-induced long-term signs of alteration of neurological and neurophysiological functions. Thus, it will be necessary in the future to find new markers describing noncholinergic outcomes of low-level nerve agent exposure.

The long-term behavioral toxicity of nerve agents, especially the alteration of cognitive functions (T-maze, Y-maze, Morris maze test) due to nerve agent-induced delayed toxicity, seems to be connected with the neuropathological damage observed in the hippocampus. Thus, neuropathology of the hippocampus connected with the alteration of cognitive functions can occur after high-level as well as repeated or long-term low-level nerve agent exposure.

Neurochemical analysis of repeated or low-level nerve agent exposure provokes the suggestion that the prolonged nerve agent-induced alteration in brain chemistry may be a pharmacological basis for neurobehavioral changes. Thus, it is necessary to follow brain homeostasis during acute as well as chronic nerve agent exposure.

Repeated or long-term exposure to low levels of nerve agents can cause neurophysiological and behavioral alterations due to downregulation of muscarinic receptors in the hippocampus as a reaction to acetylcholine accumulation at muscarinic receptor sites based on AChE

inhibition. This phenomenon is considered to be the cause of behavior performance deficits, especially disruption of cognitive functions.

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Chapter 34

The respiratory toxicity of chemical warfare agents

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34.1 Introduction

Chemical warfare agents (CWAs) play a significant role in the defense preparation plans of major military organizations worldwide. Human CWA exposure produces unique pulmonary and systemic health effects. The exposure/response relationships have been characterized to some extent, but many of the precise mechanisms are extremely complex and unknown. The physiology, biochemistry, and toxicology of CWA exposure has been studied for decades, but more recently, the interest in this topic has been rejuvenated due to increased threat for potential use of CWAs and development of countermeasures. The health studies involve acute and chronic exposure effects to high or low doses of various agents. The landscape for the administration of medical therapies has evolved from these studies. However, recent history has shown that some of the focus of chemical defense has shifted to the biological agents that have attracted much attention with the deadly anthrax attacks in the early part of this century. While many of these agents pose a different kind of threat with regard to their potential use, this chapter deals specifically with the more common CWAs, including traditional nerve agents such as sarin, soman, and tabun; vesicants such as sulfur mustard; riot control agents (RCAs); metabolic poisons such as cyanide; and choking agents such as phosgene and chlorine. Many of the agents discussed here, while not necessarily regarded as valid CWAs in current plans and scenarios, remain legitimate terrorist weapons.

In an attempt to elucidate the site-specific targets of toxicity and to define the physiological, biochemical, and toxicological mechanisms of an inhalation exposure, rodent models have been extensively utilized. This has been the case with practically all studies that investigate the adverse effects of an inhalation challenge. While these models are extremely useful as a first attempt to identify exposure/response effects, rodents do not accurately represent humans in terms of respiratory structure and function. Therefore, the extrapolation of rodent data to humans should be made carefully with regard to the toxicity of CWAs. The agents discussed herein manifest their toxicities based on the capability of the exposed animal to distribute, detoxify, metabolize, excrete, and override the deleterious effects of an agent challenge, regardless of route of exposure. However, when the toxic load overwhelms the system, potentially adverse outcomes are imminent.

34.2 History of chemical warfare agents use

The use of CWAs on the battlefield gained significant recognition during and after World War I. An excellent and comprehensive history of the use of CWAs is presented by Hilmas et al. (2008) and Salem and Tourinsky (2008). The historical use of CWAs stems from work with plant poisons by the ancient Egyptians and Indian civilizations nearly 5000 years ago. One of the earliest recorded uses of chemical warfare was that of Greek fire, as noted in the Ebers Papyrus 3500 years ago. Greek fire may have consisted of pitch, quicklime, and sulfur naptha. Arsenic-based and phosgene-like toxic smokes and "flaming concoctions" were used throughout history by the Greeks, Chinese, and Romans before the common era (BCE) to achieve victories in such settings as the Peloponnesian Wars (431–404 BCE) against tribal

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mountain rebels, and the Turks over 2000 years ago, respectively. During the fifteenth century, Leonardo da Vinci proposed the use of shells filled with copper acetate, sulfur, and arsenic as a weapon to be fired against enemy ships to disable the crew by directly affecting their lung. Similar toxic smokes were used during the Thirty Years War (1618–48). The use of chlorine gas was suggested during the US Civil War (1861–65), but it was not actually manifested under battlefield conditions until World War I. By far, the most extensive use of CWAs on the battlefield occurred during World War I. Both Germany and the Allies used projectiles filled with chlorine, phosgene, diphosgene, and sulfur mustard. These were used singly or in combinations, such as chlorine used with phosgene. These agents were well suited for use, as they generally tended to be heavier than air, and as such, caused extensive mortality and morbidity. In the mid-1930s, during the Italian-Ethiopian War, sulfur mustard (and possibly chloropicrin (PS) and phosgene) were used. In the intervening years between World War I (1914–18) and World War II (1939–45), significant effort was put into developing therapies and physical barrier protection, such as masks and suits, for the possibility of the chemical warfare attack by warring nations in future conflicts. Although CWAs were stockpiled during World War II, none were used. The development of more deadly chemical agents—namely those of the organophosphorus nerve agents—occurred during this period.

In the 1960s, during the Vietnam War, the United States used defoliating agents such as Agent Orange to uncloak the enemy. While the planned use of sulfur mustard was not applied on the battlefield, the United States implemented the use of nonlethal RCAs. In the Yemen Civil War, the Egyptians used mustard gas, phosgene, nerve agents, and RCAs on a regular basis from 1963 to 1967. In the mid-1980s, sulfur mustard and nerve agents were used by the Iraqis on Iranian troops, causing massive casualties and deaths. Over 35 years later, the survivors of these gas attacks continue to have exposure-related health issues. More recently, in 2002, the Russians used the incapacitating agent fentanyl to subdue Chechnyan terrorists holding Russian civilians hostage in a movie theater. This attempt to defuse the situation resulted in over 118 deaths due to asphyxiation. In 2007, terrorists exploded homemade chlorine canisters, causing numerous casualties in Iraq. Even more recently, in 2013, there was the alleged use of the nerve gas sarin by the Syrian government in the Syrian Civil War that presumably killed hundreds of civilians. Virtually all these agents, regardless of their route of exposure, can injure the respiratory system.

34.3 The respiratory system

The structure of the respiratory system is complex, so the effects produced by inhaled CWAs can vary depending on the chemistry and deposition efficiencies of these substances. The respiratory system is comprised of the airway compartment, to transport air, and the alveolar compartment, to perform the gas-exchange function. The airway begins with the nose, which through the pharynx carries air to the trachea, divides into two main bronchi and then several small bronchioles within each lung. Epithelial cells of different types line the airways and perform cell-specific functions. The pseudostratified, columnar ciliated cells predominate in the airways, and while guiding the airflow, function to remove particles encountered through inhaled air. Dome-shaped, secretory cells within small airways (known as *Clara cells*) secrete uteroglobin, possess drug-metabolizing enzymes, and function as stem cells to replenish lost ciliated epithelial cells. In addition, goblet cells produce and secrete mucus at the apical surface of the airway epithelium. The mucous layer helps remove particulates and pathogens via mucociliary clearance and modulates the innate immune response. The thickness of the mucous layer is proportional to the diameter of the airway and the density of goblet cells in a given area. At the basal sector of the airway epithelial cells is the basement membrane, which supports the epithelial cells and interstitial space and allows communication to the smooth muscle layer. Dendritic cells that project between airway epithelial cells function as antigen-presenting cells which, upon recognition of antigens (particulate, microbial, or soluble substances), produce innate and humoral responses (Cook and Bottomly, 2007). Sensory, vagal C-fibers innervate the airways at the airway epithelium and evoke a classical reflex reaction when stimulated, leading to bronchoconstriction and coughing, which in turn enhances parasympathetic tone, causing bradycardia and hypotension (Pisi et al., 2009). The airway surface layer is the first to encounter inhaled soluble and insoluble respirable toxicants that pass through the nose after inhalation.

The terminal airways, called *respiratory bronchioles* in humans, lead into small alveolar sacs, which are lined with type 1 and type 2 alveolar epithelial cells. Thin type 1 epithelial cells cover the alveolar surface, while cuboidal type 2 cells function to secrete proteins and surfactant material that is stored in lamellar bodies. The pulmonary arteries, which bring blood from the heart to the lung for oxygenation, form a capillary network surrounding the alveolar sacs. Type 1 cells are close to the capillary walls, allowing the diffusion of carbon dioxide from the blood to the air and oxygen from the air to the blood to occur (Galambos and Demello, 2008; Herzog et al., 2008). Myofibroblasts are found within the interstitial tissue supporting the alveolar compartments and capillary network and are involved in various functions, including synthesis of collagen, elastin, and other extracellular matrix proteins. The alveolar sacs, interstitial matrix, and capillary

network are encapsulated by the pleural mesothelial layer, which provides anatomical structure to each lung.

The surfactant material produced within type II cells is composed of about 80% phospholipids and about 20% neutral lipids and proteins. Once secreted, it layers thinly over the entire alveolar epithelial surface and provides stability to the alveoli, prevents collapse, and preserves patency. In addition to maintaining surface tension, surfactants play an important role in host defense. Of the four surfactant proteins (SPs), SP-A, SP-B, SP-C, and SP-D, SP-A and SP-D play a role in host defense, whereas hydrophobic SP-B and SP-C are involved in the adsorption and spreading of the surfactant material along the alveolar lining (Griese, 1999; Enhorning, 2008). Alveolar macrophages within the alveoli protect the lung from inhaled pathogens by engulfing bacteria, particles, damaged surfactants, and dead neutrophils or other cells after an acute lung injury. In addition, macrophages perform important innate immune functions by expressing cytokines that are involved in mounting an inflammatory response, as well as in the resolution of inflammation. A variety of warfare agents and pathogens can produce acute lung injury and alveolar edema and lead to respiratory collapse when exposures are encountered at high levels.

34.4 Pulmonary agents

Practically all CWAs can be classified as airway and lung toxicants. When inhaled, CWAs can penetrate to various levels of the respiratory system, from the nasal passages to the lung periphery, and cause toxicities specific to lung cells. More importantly, the development of many CWAs were specifically designed to cause significant mortality and morbidity due to compromised respiratory function. These include phosgene, chlorine, diphosgene, and chloropicrin, all of which were used either singly or in combination during World War I and other conflicts worldwide. While, these compounds may be found in old agent stockpiles, both phosgene and chlorine pose industrial and occupational hazards because they are also employed in many industrial chemical manufacturing processes. They are also toxic combustion by-products. Their heavy industrial usage classifies them as toxic industrial compounds (TICs), with the significant potential to affect large residential or rural areas if exploited. In this chapter, we describe the respiratory toxicity of the known warfare agents for which inhalation is the primary route of exposure.

34.4.1 Arsine

Arsine (also known as *arsenic trihydride, arsenous hydride*, and *hydrogen arsenide*) has been described as the most toxic form of arsenic. Its chemical formula is AsH_3, and it has a molecular weight of 77.95. It is 2.5 times as dense as air, which makes it highly suitable for a "state-of-the-art" trench-type warfare agent. Arsine most likely would now be classified as a low toxicity and high persistence CWA, at a level equivalent to chlorine (Table 34.1). However, due to its low toxicity (10 times less toxic than phosgene), its battlefield usage was halted during World War I. It is colorless and can have the mild odor of garlic. AsH_3 is soluble in chloroform and benzene, but only slightly soluble in water. Currently, arsine gas is used in the microchip industry to plate semiconductors with arsenic. Other workplace activities where arsine is used are for galvanizing, soldering, etching, and lead plating. Arsine is a strong reducing agent and can be formed through the following arsenide, Zn_3As_2, and acid hydrolysis reaction (Eq. 34.1):

$$Zn_3As_2 + 6H^- \rightarrow 2AsH_3 + 3Zn^{2+} \qquad (34.1)$$

34.4.1.1 Exposure physiology

The single most important route of exposure to arsine is through inhalation. Generally, no discomfort occurs from exposure to arsine, as it is a nonirritating compound. The extent and nature of the symptoms depend largely upon the concentration and duration of exposure. Romeo et al. (1997) report that exposure levels of 10−32 mg/m^3 for up to several hours may produce symptoms consistent with arsine toxicity (described next). An earlier study (Morse and Setterlind, 1950) indicated that exposures ranging

TABLE 34.1 Classification of CWAs and their military symbols based on environmental persistence and lethality.

Potency	High persistence	Low persistence
Low lethality	Sulfur mustard (HD), lewisite (L), mustard-lewisite (HL), nitrogen mustard (HN1−3), cyanogen chloride (CK), diphenylchloroarsine (DA), arsine (AsH_3), chlorine (Cl_2)	*o*-Chlorobenzyl-malonitrile (CS), dibenz(*b*,*f*) − 1,4-oxazepine (CR), chloracetophenone (CN), chloropicrin (PS)
High lethality	Soman (GB), cyclosarin (GF), VX	Phosgene (CG), sarin (GB), tabun (GA), hydrogen cyanide (AC)

from 23 to 970 mg/m^3 may be lethal to humans. Many symptoms are latent in nature. Within 1−24 h after inhalation at a high concentration, persons become ill and may experience hematuria (the voiding of dark, bloody urine). Massive hematuria may lead to anuria, which can become fatal. Within 24 h, symptoms may also include headache, vomiting, muscle weakness, dyspnea, nausea, confusion, wheezing, and jaundice. Fowler and Weissberg (1974) have shown that arsine causes tachycardia, tachypnea, hepatic enlargement, and abdominal rigidity and tenderness. In severe cases, acute respiratory distress syndrome (ARDS) could become evident along with hyperthermia, hypotension, and paresthesias in the extremities. According to the Centers for Disease Control and Prevention (CDC), high levels of inhaled arsine can also produce convulsions, loss of consciousness, paralysis, and respiratory failure, ultimately leading to death. Pulmonary edema and circulatory collapse may also result in a fatal outcome. However, at low concentrations of 10 ppm for 6 h/day for 4 days, arsine exposure to pregnant mice and rats resulted in no developmental effects (Morrissey et al., 1990). The chronic effects of arsine poisoning include renal injury, polyneuritis, memory loss, and agitation. The most significant finding of arsine toxicity is the fulminant lysis of erythrocytes. It has been reported that arsine at estimated concentrations of 750−1500 ppm × min will cause extensive hemolysis (Caravati and Dart, 2004). The LCt_{50} for arsine gas is estimated to be 5000 mg × min/m^3 (Seto, 2011).

34.4.1.2 Exposure biochemistry

The effect of arsine on the blood has been studied in several animal inhalation exposure models. Blair et al. (1990a,b) exposed male and female mice to arsine at concentrations totaling 9−900 ppm administered for 6 h/day for 5 days over 13 weeks. Pathophysiological results at the highest total dose showed significant decreases in hematocrit (HCT) and hemoglobin (HGB) with increases in mean corpuscular hemoglobin at 5 days postexposure. Between 15 and 90 days, a regenerative process appeared to have been activated by elevated reticulocytes and mean corpuscular volume. At 90 days, denatured proteins such as Heinz bodies were present. Also at 90 days, the formation of methemoglobin suggests that the oxidation of ferrous heme iron (Fe^{2+}) to ferric heme iron (Fe^{3+}) had occurred. When red blood cells (RBCs) were exposed to arsine gas in vitro, reduced glutathione (GSH) levels were diminished by 60% after a 4-h exposure (Blair et al., 1990a,b). The authors concluded that an enhanced oxidative environment may be the source of the oxidation of membrane-bound sulfhydryl groups on the HGB molecule. This conclusion was corroborated to some extent by the addition of the sulfhydryl inhibitor *N*-ethylmaleimide, 2 h after arsine exposure, which resulted in less hemolysis (Rael et al., 2000). Ionic gradient hemostasis is also affected by arsine exposure in RBCs. Rael et al. (2000) have shown that intracellular K^+ and Mg^{2+} decreased, whereas Na^+, Cl^-, and Ca^{2+} increased. Based on these data, the increase in Ca^{2+} influx may be responsible for arsine-induced hemolysis of RBCs. However, the role of oxidized GSH and an amplified oxidizing environment has been questioned by some investigators as the primary reason for arsine-induced hemolysis (Hatlelid et al., 1995; Winski et al., 1997). Pulmonary toxicity of arsine has not been reported, although inhalation exposures have been used in many studies, except for one where intraperitoneal (i.p.) injections of dimethylarsine and trimethylarsine have resulted in lung tumor formation (Yamanaka et al., 2009).

34.4.1.3 Exposure histopathology

A search of literature did not reveal any lung pathological reports for arsine gas toxicity. As stated earlier, the most prominent event following an inhalation challenge is erythrocyte hemolysis.

34.4.2 Chlorine

Similar to phosgene, chlorine is considered a "choking" agent. Under ambient conditions, chlorine, also known as *dichlorine* (molecular weight/MW = 71), is a pungent, noncombustible, yellow-green gas. Chlorine is heavier than air, which enables it to settle in low-lying areas, making it a possible persistent agent, and yet it does not have the toxicity of phosgene (Table 34.1). It is slightly soluble in water in which it can form hydrochloric and hypochlorous acids (HOCl). Chlorine can also bind with alkenes, alcohols, and ammonia. Due to high electronegativity, chlorine can react with numerous biomolecules present in cells and tissues. Reactions with Cl_2 can occur by either radical or ionic processes that form one or two chlorine atom bioproducts. As a nonmetal halogen, Cl_2 is not formed in nature due to its high reactivity. After fluorine and oxygen, it is the third most electronegative element. It was one of the first widely used CWAs, having been employed early in World War I. Presently, the majority of exposures to chlorine occur through accidental occurrences such as train derailments, industrial process failures, and recently, the intentional use of these substances by terrorists in Iraq in 2007 (Weill, et al., 1969; Hilmas, et al., 2008; Van Sickel et al., 2009).

34.4.2.1 Exposure physiology

The principal route of exposure to chlorine is through respiration. Chlorine is classified as a pulmonary irritant that can affect both the central and peripheral airway compartments. Unlike phosgene, chlorine may not obey Haber's

rule; that is, that a constant concentration × time ($[C] \times t$) product leads to a consistent physiological/toxicological outcome (Hoyle et al., 2010). Acute lung injury is not necessarily caused by chlorine itself, but by the aqueous reaction products formed in the mucus membrane of the airways. These include chlorine dioxide, chloramine, and HOCl. General sequelae include, but are not limited to, lacrimation, rhinorrhea, conjunctival irritation, cough, sore throat, laryngeal edema, dyspnea, stridor, pulmonary edema, and ARDS, all of which can lead to respiratory collapse. Furthermore, inhalation leads to abrupt airway bronchoconstriction, increased airway resistance along with decreased compliance, epithelial cell necrosis, and microvascular permeability. Chlorine exposure has also been linked to irritant-induced asthma, or what is commonly known as *reactive airways dysfunction syndrome (RADS)*. RADS is characterized by airway hyperreactivity, fibrosis, and airway obstruction. Thus, the responses to chlorine are generally nonspecific, as they are with most irritant compounds. The LCt_{50}, the amount of chlorine inhalation needed to cause 50% mortality in exposed animals or humans, is estimated to be 19,000 mg × min/m^3 (Seto, 2011).

To understand the physiological and mechanistic responses to chlorine inhalation, exposure-response studies have been conducted in rodents. Acute respiratory responses following exposure of female mice to chlorine were examined by Morris et al. (2005). In this study, mice were exposed for 15 min at total chlorine concentrations ranging from 12 to 57 ppm × min. It is not surprising that in obligate nose-breathers, the results indicate that chlorine was scrubbed with excellent efficiency in the upper respiratory tract (URT) at 97%. Resistance in the URT beginning at 15−17 days after exposure was also significantly higher with chlorine than with deep lung irritants. The RD_{50} (the 50% reduction in respiration rate as measured by frequency), at 57 ppm/min of chlorine was significantly increased from baseline to twofold during the last 6 min of 15 min total exposure. Mo et al. (2013) reported an impairment of lung antimicrobial activity in male mice exposed to a total concentration of 14,400 ppm × min chlorine and then challenged with *Aspergillus fumigatus* 24 h postexposure. In these mice, significant increases in the recruitment of lymphocytes, monocytes, and neutrophils were measured compared to chlorine exposure alone. The authors show that increased neutrophils and concentrations of reactive oxygen species (ROS) may be responsible for the inability of exposed mice to mount an immune challenge to *A. fumigatus*.

34.4.2.2 Exposure biochemistry

Chlorine inhalational damage is not restricted to particular cell types, such as epithelial cells. Injury caused by inhaled chlorine can be complex and may involve multiple pathways. The loss of vascular tone following chlorine exposure has been linked to dysfunctional nitric oxide (NO)−dependent mechanisms and resulting vasodilation (Honavar et al., 2011). To address the role of NO, Honavar et al. (2014) found that when rats were exposed to a total chlorine concentration of 12,000 ppm × min, isolated pulmonary artery studies showed disruption of vascular tone due to disrupted NO signaling. The balance between endothelial nitric oxide synthase (eNOS)- and inducible nitric oxide synthase (iNOS)-derived NO was disrupted by chlorine. The expression and activation of eNOS and iNOS through the interaction between p38 MAPK- and PI3/AKT-dependent pathways may also have an effect on endothelial permeability in lung injury. However, the precise mechanism by which eNOS and iNOS might be linked to functional impairment of pulmonary vascular tone, and bronchoconstriction is not well understood.

Exposure to chlorine in mice induces inflammatory pathways, resulting in the recruitment of neutrophils and the production of cytokines such as GROα/CINC/KC, IL-6, and TNFα (Tian et al., 2008; Song et al., 2011). Li et al. (2013) have determined that exposure of mice to chlorine gas, 400 ppm × 30 min upregulates unfolded protein response (UPR) in the lung for up to 6 h postexposure. UPR elements are regulated by a defensin-like peptide through the disruption of iron homeostasis. The authors speculate that inflammatory mediators such as TNFα, IL-6, and hepcidin might also be involved. The link between UPR elements and inflammation has been demonstrated in other studies (Xue et al., 2005). Chlorine exposure also affects regulatory Na^+ channels in epithelial lung cells (Lazrak et al., 2012). Epithelial Na^+ channels (ENaC), which are present on the apical surface, were inhibited in mouse lung slices and Type II epithelial cells after exposure to 400 ppm × 30 min of Cl_2. The data suggests that Cl_2-activated ERK1/2 expression (extracellular signal-related kinase) in Type II cells in vitro and in vivo might be involved. The data suggests that Cl_2 exposure results in compromised Na^+ regulatory activity, partly responsible in fluid clearance mechanisms. Postexposure treatment with reactive species scavengers ameliorated the problem (Xue et al., 2005). The common thread among these studies is that chlorine-induced inflammation produces reactive intermediates and induces cytokine release, which can ramp up destructive pathophysiological responses. Chloramine, a reactive by-product of chlorine metabolism, is also capable of initiating reactive processes. The degree of chlorine-induced injury and lung inflammation has been shown to differ between mouse strains (Tian et al., 2008).

34.4.2.3 Exposure histopathology

In humans who have died from severe chlorine exposure, postmortem results have shown the presence of massive pulmonary edema, ulcerative bronchiolitis, and

cardiotoxicity (White and Martin, 2010). In mice exposed to chlorine at 400 ppm × 15 min, profound changes in lung pathology were observed (Hoyle et al., 2010). This study looked at lung damage through a range of concentrations over various points in time that produced a constant concentration × time ($[C] \times t$) product. There was significant damage to the airway epithelium. At 6 h postexposure, the injury was more widespread, encompassing large airways and causing denudation. At 24 h postexposure, sloughed epithelial tissue fragments were present in the airway lumen. There was also evidence of neutrophilic inflammation that is consistent with inhaled chlorine toxicity. The magnitude of the histopathological response was not necessarily $[C] \times t$-dependent. These data corroborate a comparable study by Tian et al. (2008) where similar histological responses were observed. However, at a later time point after exposure (48 h) repair processes are likely activated. Thus, exposure to high concentration of chlorine can produce profound lung injury and pathology.

34.4.3 Phosgene

Phosgene (also known as *carbonyl dichloride, carbon dichloride oxide, carbon oxychloride, chloroformyl chloride, dichloroformaldehyde*, and *dichloromethanone*), whose chemical formula of $COCl_2$ (MW = 98.9) was used as a CWA during World War I. In addition to its weapons applications, it has a variety of industrial uses, such as in the synthesis of pharmaceuticals and organic materials (Wyatt and Allister, 1995). Because of its potential for toxicity, phosgene is often produced and used captively in the same chemical facility to avoid potential hazard. Although it is less toxic than nerve gases such as sarin and tabun, it is still regarded as a viable CWA because it is so easy to produce. Phosgene is generally produced in industry by the interaction of carbon monoxide and chlorine gas, using activated carbon as a catalyst. It can also be produced from chloroform in the presence of oxygen and ultraviolet (UV) light. When phosgene reacts with water, it decomposes into hydrogen chloride (HCl) and carbon dioxide. Accidental exposures to phosgene in humans at high concentrations cause pulmonary edema, sensory irritation, and associated rapid breathing (Diller, 1985a). Patients complain of dyspnea upon exertion and reduced ability to exercise for several months to years after an accidental exposure. Normalization of lung function can take several years (Diller, 1985b).

34.4.3.1 Exposure physiology

Phosgene can be widely and easily dispersed by the wind. Being heavier than air, it tends to sink into trenches and ditches making it fairly nonpersistent on the ground (Table 34.1). Phosgene is deposited in deeper regions of the lung, as opposed to nitrogen oxide, sulfur oxide, and chlorine, which are more water-soluble and are deposited primarily in the upper airways. Inhaled phosgene causes chest pain, burning throat, and persistent cough. It affects numerous metabolic pathways critical to cell and tissue survival. The average LCt_{50} of phosgene in acutely exposed rats has been reported to be 1741 $mg/m^3 \times min$ (Pauluhn, 2006a). The respiratory toxicity of phosgene is well studied in laboratory rodents, and large animal species such as dogs and sheep. There is a steep acute $[C] \times$ time mortality relationship for phosgene gas in rats (Pauluhn et al., 2007).

Exposure to phosgene was associated with early bronchoconstriction, an obstructive injury pattern, and disruption of mechanical rhythm of breathing, which were largely attributed to the progressive production of pulmonary edema in mice (Sciuto et al., 2003). Increased expiratory resistance and decreased dynamic compliance were noted in rats exposed to phosgene (Ghio et al., 2005). In pigs exposed to a high concentration of phosgene, a transient decrease in oxygen saturation and cardiac stroke volume index was observed during the exposure period, while significant decreases in arterial pH, P_aO_2, and lung compliance were noted 6 h after exposure (Brown et al., 2002). Thus, the acute physiological effects are likely mediated by sensory irritation and lung edema.

34.4.3.2 Exposure biochemistry

Once inhaled, phosgene penetrates deep into the alveolar region, where it transits through the airway surface and is hydrolyzed to CO_2 and HCl; however, it has been shown that the concentration of HCl is not sufficient enough to produce the damage that is typically seen after an acute phosgene exposure (Pauluhn et al., 2007). In contrast to soluble gases, the less-soluble phosgene gas penetrates the lower respiratory tract without marked retention in the conducting airways. It has been hypothesized that phosgene-induced acylation of nucleophilic amino, hydroxyl, and sulfhydryl moieties of the fluid components of the alveolar lining, rather than HCl itself, results in rapid alveolar injury and inflammation (Pauluhn et al., 2007). Through this acylation reaction, phosgene instantaneously interacts with antioxidants such as GSH, resulting in its depletion (Sciuto et al., 2003, 2005). It has been proposed that surfactant destabilization and generation of reactive oxidation by-products results in rapid intra-alveolar pulmonary edema. The surfactant abnormalities have been presumed to initiate events leading to acute respiratory failure (Mautone et al., 1985).

Many experimental studies have investigated transient surfactant abnormalities soon after phosgene exposure (Jugg et al., 1999). The surfactant, a mixture of lipids and

proteins, performs an important function of maintaining surface tension between air liquid interphase within the alveoli and prevents alveolar collapse. It also regulates innate immune response and host defense (Wright, 2003). Proteins leaked from the vasculature into the alveoli due to phosgene-induced damage to the surfactant and alveolar cells can further inactivate surfactant material. Upon exposure to phosgene, increases in the bronchoalveolar lavage fluid (BALF) levels of proteins are attained on day 1, while lipids increase on day 3, suggesting that these proteins are the likely cause of surfactant destabilization, leading to further vascular leakage, inducing apoptosis, and inflammatory signaling cascade via an innate immune response (Pauluhn et al., 2007). Evidence from studies using large animal models such as dogs and pigs suggests that phosgene inhalation causes a high-permeability type of lung edema brought on by high surface tension and the compensatory interstitial perimicrovascular hydrostatic pressure, resulting in alveolar flooding (Pauluhn, 2006a,b). It has been postulated that the latency between phosgene entry into the lung and edema are due to the time required for increased fluid distribution between interstitial, lymphatic, and perimicrovascular compartments within the alveoli. The alveolar epithelial and interstitial cells (but not endothelial cells) are the primary targets of phosgene-induced injury, further supporting the hypothesis that pulmonary edema results from a pressure gradient across the air liquid interphase (reviewed in Pauluhn et al., 2007).

Increased pulmonary edema induces a sequence of events that results in the release of inflammatory cytokines, apoptosis, extravasation of inflammatory cells, and inflammation that is linked to extracellular matrix remodeling and fibrosis observed in a number of studies, specifically those involving low-level, longer duration exposures. The degree of inflammation and subsequent fibrosis could be directly related to the concentration of phosgene, the longevity of exposure, and the type of laboratory animal model being exposed (Kodavanti et al., 1997; Pauluhn et al., 2007). The removal of pulmonary liquid and inflammatory cells may facilitate quick reestablishment of the homeostasis. A number of processes, such as mucociliary clearance, fluid clearance through pulmonary circulation, and inflammatory cell signaling, are initiated within alveolar and lower airway structures. Although acute pulmonary toxicity of phosgene is fairly well established, there are no effective antidotes other than supportive management of symptoms. A number of different therapeutic approaches have been tested experimentally (Sciuto and Hurt, 2004).

34.4.3.3 Exposure histology

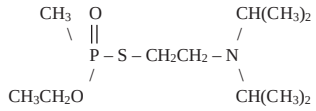
Histopathological lesions have been reported after an acute exposure to phosgene, which are characterized by alveolar and interstitial edema, hemorrhage, fibrin deposition, alveolar and interstitial flooding, and inflammatory cell infiltration. Focal bronchiolar and terminal airway degeneration and necrosis have also been reported. Resolution of inflammation and edema have been noted in a number of animal studies (Gross et al., 1965; Hatch et al., 2001; Duniho et al., 2002). At relatively low concentrations and longer exposures, phosgene can induce alveolar inflammation and fibrosis in a concentration-dependent manner (Kodavanti et al., 1997). The histologic changes in the bronchioalveolar regions in rats exposed to phosgene at 0.1 ppm for 4 weeks were characterized by a small but apparent thickening and mild inflammation, which were progressive with increased concentrations. Masson's trichrome staining indicated increased collagen deposition at the terminal bronchiolar sites and increased pulmonary hydroxyproline, a measure of collagen deposition, at high concentration of 1 ppm. These lesions were also seen in animals exposed for 12 weeks and persisted after a 4-week nonexposure recovery period. Histological changes, including collagen deposition after long-term phosgene exposure, have also been reported by Pauluhn (2006a). Histopathology in pigs exposed to high concentrations of phosgene revealed areas of widespread pulmonary edema, petechial hemorrhage, and bronchial epithelial necrosis (Brown et al., 2002). Thus, phosgene-induced injury is likely to cause pulmonary fibrosis and scarring of the peripheral lung tissue, which might affect breathing.

34.4.4 Nerve agents

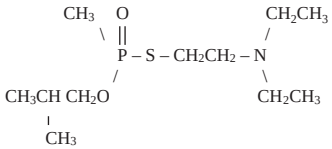
The nerve agents consist of a family of compounds whose role in chemical warfare was to cause significant morbidity and mortality among soldiers. As such, the most likely application of nerve agents was initially for military purposes. However, as recently as 2013, nerve agents were allegedly used against civilians in conflicts in the Middle East (Sellstrom et al., 2013). The syntheses and development of nerve agents took place in Germany between World War I and World War II. Nerve agents are highly toxic and are classified as organophosphates (OPs). OPs are esters of phosphoric acid and are the most toxic of the known chemical CWAs. Members of this family also include parathion, malathion, and mipafox, which are commonly used worldwide in agriculture as well.

The principal chemical varieties of chemical warfare nerve agents are shown in Fig. 34.1. Nerve agents are generally odorless and colorless. See Table 34.2 for physical chemical characteristics. Sarin (GB), tabun (GA), cyclosarin (GF), and soman (GD) are known as the *G agents*, where *G* denotes "Germany." These agents volatilize fairly rapidly. Whereas the *V agents* (where *V* denotes "venomous"), such as *O*-ethyl *S*-[2-(diisopropylamino)ethyl]

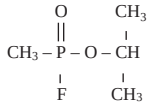
A. VX (*O*-ethyl *S*-(2-(diisopropylamino) ethyl) methlyphosphonothioate)



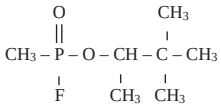
B. VR (*O*-isobutyl *S*-[2-(diethylamino)ethyl] methylphosphonothioate)



C. Sarin ([GB], isopropyl methylphosphonofluoridate)



D. Soman ([GD], pinacolyl methylphosphonofluoridate)



E. Tabun ([GA], ethyl *N*-dimethyl phosphoroamidocyanidate)

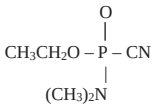


FIGURE 34.1 Chemical structures of nerve agents.

methylphosphonothioate (VX) and VR, are classified as nonvapor hazards. VR (also known as *Russian VX*) is a close stereoisomer of VX. Classifying V agents as nonvapor threat agents may not be entirely appropriate, as these agents can penetrate the airways if they adhere to dusts, mists, and fog particles, thereby causing significant effects. As a class of threat agents, they are meant to be used as offensive weapons and can be delivered via rockets, bombshells, mortar rounds, and other devices. Usually dispersion is in the forms of sprays, aerosols, and vapor, and possibly the combination of aerosol and vapor. The toxicity response produced by a mixture of OPs can be multifaceted with respect to how these agents are absorbed and deposited in the airway. Punte et al. (1958) makes the statement that OPs in the aerosol form may be more toxic than an equivalent amount delivered by a gas exposure. Owing to their rapid uptake, nerve agents can be absorbed by inhalation, dermal, ocular, and oral routes of exposure. The neuronal effects of nerve agent intoxication include physiological, toxicological, and biochemical responses, which are well studied, but the pulmonary effects are less well examined. These effects are common to all the agents with structural similarities, as shown in Fig. 34.1.

Regardless of the route of exposure, nerve agents cause a variety of physiological and toxicological effects, as discussed next. Many of these occur within minutes of exposure, especially with the more volatile G agents. Studies involving inhaled nerve agents have been conducted for many years with a range of animal models. Many factors affect the toxicity response—for example, whole-body exposure versus head-out or nose-only, animal species, agent type, time of day of exposure, duration of exposure, aerosol versus vapor (as these enter the airspaces at different rates and in different locations), dilution vehicle used, agent concentrations and particle size (in the case of an aerosol), and most important, the target tissue dose. Pulmonary toxicities of different classes of nerve agents are described next.

34.4.4.1 Volatile agents

G agents include GA, also known as ethyl dimethylamidocyanophosphate, EA1205 (tabun); GB, known as isopropyl methylphosphonofluoridate, trilone, MFI, TL1 618, T144, and T2106 (sarin); and GD, known as pinacolyl methylphosphonofluoridate (soman). They are among the most toxic CWAs. Listed in descending order of ease of volatilization, it is GB, GD, and GA. GA is colorless and may have a fruity odor, which can change to bitter almonds (similar to cyanide) upon decomposition. GA is soluble in organic solvents such as ethanol, diethyl ether, and chloroform. Hydrolysis of GA can produce cyanide and hydrocyanic acid. GA has a $t_{½}$ of 8.5 h at a pH of 7 in an aqueous solution. GB is colorless and odorless, and it is the most volatile of the G agents. It is miscible in water and, like GA, is hydrolyzed in both acidic and basic conditions. This rapidly acting compound has a $t_{½}$ of about 1.5 min. at a pH of 11 and 25°C. However, one report shows an estimated $t_{½}$ to be 40 h (Garigan, 1996). GB comes in two enantiomeric forms (+) and (−), with the (+) form twice as toxic as the (−) form (Christen and Van den Muysenberg, 1965). GD is also a colorless liquid with a fruity odor. It is about 20% soluble in water at 25°C. In water at 20°C, and a pH of 7, it hydrolyzes with $t_{½}$ of about 80 h. GD has two isomers, C(±)P(−) is more toxic than C(±)P(+), each possessing different rates of hydrolysis, with C(±)P(+) being faster. GF and GA are the least studied of the G agents and will not be discussed herein. Chemical properties of the G agents are shown in Table 34.2.

34.4.4.2 Exposure physiology

Considerable valuable and relevant data have come from many experimental models using the inhalational approach to assess G agent toxicity. The organ distribution of G agents can take on different patterns following exposure with regard to the volume of distribution. GB is distributed to the brain, kidney, liver, and blood plasma of mice (Little et al., 1986). Within the brain, both GA and

TABLE 34.2 Nerve agent physicochemical properties.

Property	Sarin (GB)	Soman (GD)	Tabun (GA)	VX/VR
Molecular weight	140.1	182.2	162.1	267.4
Specific gravity at 25°C	1.0887	1.022	1.08	1.0083
Melting point (°C)	−56	−80	−50	−39-calculated
Boiling point (°C)	147	167	245	300
Vapor pressure (mmHg)				
0°C	0.52	0.044	0.004	
10°C	1.07	0.11	0.013	
20°C	2.1	0.27	0.036	0.00044
25°C	2.9	0.4		0.0007
30°C	3.93	0.61	0.094	
40°C	7.1		0.23	
50°C	12.3	2.6	0.56	
Guinea pig LD_{50}, mg/kg body weight, s.c.[a], at 24 h	42	30	117	8/11.3

Missing values indicate lack of information available.
[a]*s.c., subcutaneous administration.*
Source: Adapted from Maynard, R.L., Chilcott, R.P., Ballantyne, B., Marrs, T.C., Syversen, T., In general and applied toxicology—toxicology of chemical warfare agents. In: B. Ballantyne, T.C. Marrs, T. Syversen (Eds.), In General and Applied Toxicology—Toxicology of Chemical Warfare Agents. Wiley, River Street, Hoboken, NJ, 2009, pp. 2875–2911 (Maynard et al., 2009).

GD are detected in the hypothalamus (Wolthis et al., 1986). GD can partition into fat, which delays the morbidity response in animals. In addition to distribution, elimination pathways of nerve agents consists of covalent binding and enzymatic hydrolysis. A number of studies have examined the toxicity of these agents. Van Helden et al. (2004) exposed marmosets and guinea pigs to low vapor concentrations of GB, 7–150 μg/m^3 over 10–300 min, to determine the earliest physical response to exposure. Results showed that GB, at levels that were undetected by the fielded alarm systems used, produced significant neuronal effects in the exposed animals. This suggested that acute effects of GB can occur at very low levels. Lung microinstillation exposures of guinea pigs for 4 min to GB caused increased weight loss, lung edema, decreased O_2 saturation, decreased peak inspiratory and expiratory flows, and increased minute volume within 4 h (Conti et al., 2009). Decreased flows are indicative of increased airway resistance. High-dose inhalation exposure to GB (a total dose of 130–150 mg/m^3 × min over 10 min) caused severe bronchoconstriction in rats (Gundavarapu et al., 2014). Che et al. (2008) showed that microinstillation exposure of GB in guinea pigs caused immediate inhibition in BALF in acetylcholinesterase (AChE) activity, followed by a reversal of this effect within an hour despite continued inhibition of blood AChE. This was likely due to the high lipophilicity and rapid absorption of GB. In a 3-month study using rats exposed to GB vapor at 33–35 μg/L for 10 min daily, Allon et al. (2005) determined that there was an increased vulnerability to cardiac arrhythmias after each challenge to GB. This study pointed to the potential of delayed effects of GB exposure; however, long-term consequences of single high-dose GB exposures are not well studied. In a limited study, Husain et al. (1993) showed that mice exposed to nebulized GB at 100 mg/m^3 × min per day for 10 days developed muscular weakness of the limbs and slight ataxia on day 14. GB and GD exposure-response effects on hemodynamics and lung function were assessed in baboons following an inhalation challenge. GD at 13.14 μg/kg and GB at 30 μg/kg were vaporized into the upper airway of baboons. Both agents caused increased development of apnea due to decreased phrenic nerve signals, cardiac arrhythmias, and a decrease in mean systemic blood pressure (Anzueto et al., 1990).

In an acute in vivo guinea pig lung microinstillation model, Katos et al. (2009) demonstrated that exposure to GD produces prominent respiratory dynamic changes. At 24 h postexposure, tidal volume increased along with respiratory frequency. In contrast to the study by Conti et al. (2009), peak inspiratory flow was increased, possibly suggesting agent-specific effects on lung airflow patterns. Using a head-out vapor exposure system, Perkins et al. (2013) investigated the toxicity of inhaled GD at concentrations ranging between 520 and 1410 mg × min/m^3 in the conscious rat. All animals exposed to the higher

two doses died. Significant increases in BALF protein occurred with dose-dependent inhibition of AChE activity in the lung and brain within 24 h after challenge. Cholinergic crises were evident based on dose-dependent changes in cholinergic symptoms compared to controls. It can be presumed that the systemic effects of acute nerve agent exposure may be more likely when the RBC−AChE levels drop by 75%−80% (Sidell and Somani, 1992). Systemic OP-induced depletion of AChE could be directly responsible for increases in mortality resulting from heightened convulsive and seizurogenic activity. However, a number of studies have shown the effects independent of AChE inhibition. Willems (1981) demonstrated that in OP pesticide-exposed individuals, there was no correlation between AChE inhibition and the extent of neuronal symptoms. Moreover, an acute noninhalation exposure study with sarin, VX, and soman in male and female guinea pigs demonstrated that respiratory toxicity of these compounds did not correlate with AChE activity (Fawcett et al., 2009). Mutagenicity studies with GB and GD have shown that they do not produce adverse effects in the Ames *Salmonella*, Chinese hamster ovary, and mouse lymphoma assays. However, GA was deemed slightly mutagenic in the Chinese hamster ovary and Ames bacterial system assays (Nasr et al., 1988; Goldman and Dacre, 1989).

Thus far, human data have largely come from low-dose exposure of volunteers and from nonfatal accidental exposures. Exposed humans have experienced coughing, wheezing, rhinorrhea, and nonexertional and exertional dyspnea, along with a feeling of increasing pressure in the thoracic region. Craig and Freeman (1953) provided evidence that accidental exposure to GA or GB causes behavioral changes, sleep disturbances, fatigue, and mood changes. Indeed, the concentrations of these agents required to produce mortality in humans are not precisely known. However, the estimated LCt_{50} of GA, GB, and GD are believed to be 150, 70−100, and 40−60 mg × min/m^3, respectively.

34.4.4.3 Exposure biochemistry

The nerve agents are known to bind and functionally disable AChE regardless of the agent form (i.e., vapor or spray) or the route of exposure. The extent of AChE inhibition is a function of dose and duration of exposure, basically the product of $[C] \times t$. AChE is found at the synaptic junctions of nerve endings and is responsible for hydrolyzing excess acetylcholine (ACh), which is critical to synaptic nerve transmission. The failure to metabolize or break down ACh causes an accumulation of ACh at the nerve terminals, leading to increased cholinergic stimulation and many of the toxidromic symptoms typical of nerve agent poisoning, known medically as a *cholinergic crisis*. Increased cholinergic stimulation leads to persistent stimulation of muscarinic receptors (M_1−M_3 in the lung) within the parasympathetic neurons. In mammalian species, parasympathetic nerves innervate the airways. Muscarinic receptors belong to the G-protein coupled receptor family presenting throughout the airways. Overstimulation of muscarinic receptors initiates increased salivation, lacrimation, urination, and defecation (commonly known as *SLUD*), along with bronchoconstriction, bradycardia, increased nasal secretions, emesis, and dyspnea. Nicotinic receptors, a member of the ligand-gated ion channel family, occur in the somatic or sympathetic nervous system and are also affected by OPs. Symptoms include tachycardia, mydriasis, fasciculations, meiosis, skeletal muscle paralysis, hypertension, slurred speech, irritability, fatigue, impaired judgment, insomnia, and diaphragmatic weakness. More severe poisoning is associated with more profound central nervous system (CNS) responses such as ataxia, convulsions, seizures, and death by asphyxiation.

Neurotransmitter pathways may be directly involved in the sequelae of G agent-induced toxicity. In the CNS, G agents can also act directly on glutamate receptors. Chebabo et al. (1999) demonstrated in an in vitro model that nM amounts of GB reduced the amplitude of gamma-aminobutyric acid (GABA), which is a neurotransmitter. It has been suggested that overstimulation of glutamatergic receptors by GD in the brain may be responsible for the modulation of seizure activity (Lallement et al., 1991a,b). Affected neurotransmission pathway may have far-reaching consequences on behavior and induction of convulsions. For an in-depth discussion of nerve agent exposure-response effects and case histories, refer to the excellent overview by Sidell et al. (2008). Some investigations have centered on the effects of repeated exposure to nerve agents. Kalra et al. (2002) studied the effects of subclinical repeated inhalation nose-only exposures to GB of 0.2 and 0.4 mg/m^3 on immune cell responses in rats. GB exposure suppressed T-cell mitogenesis, conconavalin A, and anti-αβ-T receptor-dependent antibody-forming cell responses. In addition, there was a reduction in glucocorticoid production. Their data indicated that repeated exposures to GB over 5−10 days caused changes in T-cell responsiveness mediated by GB's effect on the autonomic nervous system.

Inhaled G agents cause significant respiratory problems. The respiratory effects occur within minutes and clinically can resemble a severe asthmatic attack. A nerve agent-induced death is normally linked to pulmonary dysfunction. Enhanced secretions are the result of vagal efferent activity resulting from increased ACh concentration, substance P, and vasoactive intestinal peptide release. The inhalation of a high concentration of vapor will result in loss of consciousness, apnea, flaccid

paralysis, and seizures (status epilepticus) within a period ranging from seconds to several minutes. Peak effects can occur within 30 min, followed by an asphyxiating death (Berkenstadt et al., 1991).

One of the major differences between the principal nerve agents GA, GB, and GD is associated with aging of the AChE enzyme. This AChE aging is basically a chemical reaction resulting in the complexation of the nerve agent with the AChE enzyme that prevents the reactivation of the enzyme. GB-complexed AChE ages over about 5 h, whereas GD takes only minutes (Garigan, 1996). Aging half-time estimates can vary depending on the experimental model used such as in vitro versus in vivo (Sidell et al., 2008).

In addition to binding with AChE, the detoxification process can lead to interaction of OPs with other cholinesterases such as butyrylcholinesterase (BuChE) and carboxylcholinesterase (CaChE) in the plasma and tissues. Levels of these enzymes can vary from organ to organ. In humans, CaChE is present in cells rather than in plasma, as is measured in other animal species. OPs can also interact with other enzymes such as paraoxonase and arylesterase. For a more comprehensive review of these secondary effects, see Casida and Quistad (2004).

Exposure to G agents in particular GD may change the antioxidant-to-oxidant ratio by affecting free-radical scavenging pathways that are abundant in mammals. Klaidman et al. (2003) showed that an intramuscular injection of GD in rats decreased protein sulfhydryls in the piriform cortex and the hippocampus during seizures at 1 h postexposure, whereas at 24 h, postexposure GSH levels decreased nearly 50% in the piriform cortex. These results suggest that free-radical formation may be contributing to seizurogenic pathophysiology. Furthermore, subcutaneous challenge to GD increased lipid peroxidation and formation of nitrogen oxides within 30 min of administration in rat brain, especially the hippocampus, thalamus, and medulla-pons (Jacobsson et al., 1999). Since the medulla-pons region controls respiration, it is likely that neuronal mechanisms might also contribute to respiratory effects of OPs. Despite the fact that the two models cited here do not involve the inhalation route of exposure, it is clear that once the agent crosses into the systemic circulation, similar physiological responses occur regardless of the route of exposure.

Exposure to G agents can also cause changes in transcriptional pathways. For instance, RamaRao et al. (2011) showed that GD caused perturbations in the phosphorylation levels of cAMP response element binding protein (CREB), c-Jun, and NF-κB, all of which control a variety of pathological processes. Dillman et al. (2009) demonstrated using microarrays that GD markedly modulated p38 mitogen-activated protein kinase (MAPK) and extracellular receptor kinase (ERK) signaling pathways. While these were not inhalation studies, it should be noted that once the agent enters the systemic circulation, multiple comparable side effects are initiated and propagated. Exposure to G agents causes perturbations in calcium regulatory signaling pathways, which can affect a variety of physiological responses. Destabilization of protein kinase C, which is responsible for brain ion fluxes and eventual neurotransmitter release at different rates in various brain regions, suggests that brain regions respond differently to nerve agent challenges. Exposure to GB also induces inflammatory gene expression within the lung. GB increases lung tissue expression of IL-2, TNFα, IL-1β, hypoxia-induced factor HIF-α, and eotaxin 24 h following exposure, suggesting a cytokine surge resulting from highly active inflammatory processes (Gundavarapu et al., 2014). For these and other nerve agent-induced expression changes in inflammatory processes, see RamaRao and Bhattacharya (2012).

34.4.4.4 Exposure histopathology

Inhalational effects of G agents on lung tissue following exposure can range from basically none to severe based on the $[C] \times t$ of exposure. In rats exposed to 768 mg × min/m^3 aerosolized GB, major changes in the pathology at 4 days after exposure were observed. Primary changes were seen in the epithelial lining of the lobar bronchi. Lung histology showed increases in interstitial mononuclear cells and thickening of the alveolar septa. Masses of exudate, inflammatory cell infiltration, and bronchial epithelial damage were also seen (Pant et al., 1993). In a study featuring 10-day repeated exposure to GB, on day 14 postexposure spinal cord pathology showed clear evidence of degenerative axons (Husain et al., 1993). Brain lesions and cardiomyopathy were observed in rats exposed to GD or GB in a subcutaneous injection model (Singer et al., 1987; Tryphonas and Clement, 1995). In Perkins et al. (2013), rats exposed to 4−6 min GD vapor at 600 mg × min/m^3 had compromised lung tissue pathology compared with controls. At 24 h after exposure, alveolar hemorrhage, inflammation, and histiocytosis scored twofold higher than controls. Neutrophilic exudate and alveolar destruction were also observed. An intramuscular challenge with GD showed astrocytic degeneration, neuronal necrosis, and liquefaction necrosis of the CNS. The cerebral cortex, limbic system, thalamus, and substantia nigra appeared to be targets of toxicity in the rat (Tryphonas and Clement, 1995). Pulmonary pathology changes after systemic administration of nerve agents are not well characterized.

34.4.5 Nonvolatile agents

Nonvolatile agents, such as VX and its structural isomer VR, have the same molecular weight (as shown in

Table 34.2) and are not considered to be vapor hazards. As a result, they are judged to be low inhalational threats to humans (the agents, listed in order of volatility are GB, GD, GA, VX, and VR). Most research on V agents have centered on VX, which is considered the "gold standard" against which the toxicities of all other agents (GA, GB, and GD) are compared.

VX is an odorless and colorless compound when in pure form. It is an oily liquid that is slightly soluble in water. These characteristics are largely responsible for its capacity to be classified as a persistent agent (Table 34.1). Although VX and VR are similar in structure, Collins et al. (2013) demonstrated in a rat aerosol exposure model that after a single 10-min exposure to VX or VR, the VR lethal concentration was nearly half that of VX (367 versus 632 mg × min/m^3) making it more toxic. These data support results from an earlier study by Chang et al. (2002), where they conclude that it may take higher doses of atropine to treat those exposed to VR. While there may be differences between VR and VX in terms of lethality, the physiological, biochemical, histopathological, and toxicological outcomes are very similar. Therefore, unless specifically pointed out, mostly VX data are reported. The reported estimates of lethality for VX is 40 mg × min/m^3 (Seto, 2011).

34.4.5.1 Exposure physiology

While VX and VR pose more of a percutaneous threat, exposure by inhalation should be given more serious consideration, as the agent can directly come into contact with airway surfaces when bound to mists, dust particles, fog, and other substances. As a result, entry into the airspaces can be accelerated. Exposure of airway surface can lead to almost immediate reactions culminating in respiratory failure if the $[C] \times t$ product is sufficiently high. Bronchoconstriction, a hallmark of nerve agent exposure, in animals caused by the inhaled V agents most likely is a result of mechanisms common to those of G agents. Bronchospasms may be caused by local effects on the respiratory center of the CNS, as opposed to direct effects. It is likely that more persistent agents have a tendency to deposit in the lung compartment and are slowly released over time as previously described for GD. Exposure to VX or VR produces similar hypercholinergic effects such as SLUD, incapacitation, seizures, and cardiorespiratory depression (bradycardia, dyspnea, and convulsions).

Lung exposure studies in animals using microinstilled VX have provided evidence of the nature of pulmonary effects. In guinea pigs exposed for 5 min to VX doses ranging between 50 and 90 μg/m^3, acute effects varied from an increase in lung wet/dry weight ratio with increases in the numbers of macrophages/monocytes in BALF at 24 h postexposure (Wright et al., 2006). In a similar exposure model of local pulmonary instillation of VX, Graham et al. (2006) demonstrated that BuChE activity, although inhibited at 5 min postexposure, was quantifiably present in BALF at 24 h postexposure even at the highest dose of VX. The authors speculate that the presence of BuChE, which is synthesized in the liver and circulates in the plasma, may be a marker of damaged air−blood barrier integrity at later time points. Katos et al. (2007) found a significant inhibition of AChE in the esophagus and intestine following microinstilled VX in the lung. From these studies, it is apparent that VX can localize to the gastrointestinal tract, causing an "irritable bowel-like" condition as a result of its likely clearance through the gut. In guinea pig studies investigating the long-term effects of a 10 min. VX exposure on changes in pulmonary respiratory dynamics, Rezk et al. (2007) demonstrated that end-expiratory pause (EEP); that is, the length of time between the end of expiration and the beginning of the inspiratory cycle was increased at 48 h postexposure. This study also indicated that most of the altered respiratory function returned to near control levels by day 7, except for EEP. EEP did return to normal levels by day 18. These data suggest that VX can persist in the body due to tissue-specific compartmentalization and produce long-term effects.

A direct 10-min intratracheal VX exposure of anesthetized rats, bypassing the nose (preventing neuronal translocation to brain through nose), resulted in acute lung injury and tissue damage, suggesting direct tissue-specific effect. In this work, VX aerosol was administered at 514 mg/min/m^3 and produced significant increases in BALF protein concentrations at 6 and 24 h after exposure, suggesting the prevalence of pulmonary edema. Airway resistance was significantly increased at 20 min and 6 h postexposure at all VX concentrations tested and at the two highest doses, 343 and 514 mg × min/m^3, 24 h postexposure (Peng et al., 2014). The authors concluded that VX inhalation impairs pulmonary function through an obstruction associated with increased pause, expiration time, and nearly a 50% decrease in expired flow rate, decreased respiratory rate and tidal volume, leading to a drop in minute ventilation. These are all indicators of airway flow obstruction. VX inhalation could transiently disturb lung function through airway smooth muscle spasm, paralysis, or both, and when combined with mucus oversecretion, could result in respiratory failure; however, the precise mechanisms are not well understood.

Additionally, a preliminary assessment of the VX exposure effects on cellular profiles in BALF was undertaken. Study time points were 3, 6, 24 h, and 1 and 2 weeks postexposure. Blood analyses indicated that VX exposure at 343 mg × min/m^3 increased circulating white blood cells (WBCs), erythrocytes (RBCs), HCT, HGB,

neutrophils, platelets (PLTs), and eosinophils at 6 h post-VX inhalation compared with naïve controls. Lymphocytes, basophils, monocytes (MONO), HCTs, and RBCs remained elevated at 1 week. Monocytes and PLTs continued to be elevated at 2 weeks after exposure compared to naïve controls (Sciuto, 2014). While further work is needed in this area to determine how these changes are associated with pulmonary pathology and the chronicity of the damage, these data indicate that variable effects of V agent inhalation on cell populations are not limited to acute effects. This substantiates the fact that V agents may deposit in tissues and get released over a long period of time after a single exposure. It is also possible that there may be a lag-time in the immune response induced by V agent exposure as part of a compensatory mechanism.

34.4.5.2 Exposure biochemistry

Limited published studies are available investigating the effects of V agent exposure on biochemical pathways. Similar to G agents, V agents likewise inhibit blood and lung tissue AChE activity. In the aerosolized VX rat exposure model of Peng et al. (2014), blood AChE activity was decreased 41% at 343 and 52% at 514 mg × min/m^3 24 h postexposure. Lung tissue AChE activity was also inhibited by VX. However, using a similar exposure model testing the effects of VR, AChE mRNA transcription in the lungs was up-regulated by 19% for 143 and 30% for the 286 mg × min/m^3 VR groups compared to vehicle at 24 h (Sciuto, 2014). Up-regulation of lung tissue AChE mRNA may indicate the cellular response to accumulation of nonfunctional enzyme. In addition, we observed by immunohistochemistry a positive staining for iNOS in alveolar epithelial cells together with decreased staining of surfactant D in lung tissue 24 h postinhalation exposure to 514 mg × min/m^3 VX.

In these VX aerosol inhalation studies, Western blot analyses showed that xanthine oxidoreductase, an enzyme-producing superoxide, is activated in response to VX inhalation exposure at 343 mg × min/m^3. VX inhalation also triggered IL-6 expression in rat lung tissue. In VR-exposed rats, lung lavage assays showed decreased GSH concentration and superoxide dismutase (SOD) activity, which indicates a local oxidative stress environment. Western blot analyses of lung tissue 6 h postexposure demonstrated an increased expression of xanthine oxidase, increased IL-1β expression, and activation of phosphorylation of p38 and Akt suggesting stimulation of inflammatory mechanisms.

34.4.5.3 Exposure histopathology

There is minimal if any lung damage within 24 h after VX exposure in rats. Lung and tracheal lesions were generally less severe in the 3- and 6-h VX postexposure groups than in the 24-h VX postexposure groups consistent with its delayed effects. This delay could be due to the time it takes for extravasation of inflammatory cells, such as neutrophils and macrophages (Peng et al., 2014). The tracheal lesions in these animals showed evidence of ulceration accompanied by neutrophilic inflammation and necrotic epithelial lining (necrotic membrane). Similar findings were observed in a VX-exposed guinea pig inhalation model (Nambiar et al., 2007). Peng et al. (2014) also showed that at high levels of inhaled aerosolized VX, exposure produced prominent alterations in airway and lung pathology at 24 h in the rat. A 10-min exposure to 514 mg × min/m^3 VX-induced architectural changes in the trachea and lung. The most significant findings were perivascular inflammation, histiocyotsis, alveolar exudate, alveolar epithelial necrosis, septal edema, and bronchiolar inflammatory infiltrates.

VX exposure also causes lung parenchymal pathology. VX-induced histological changes are persistent for 1−2 weeks postexposure. In general, the pulmonary histologic changes described are likely secondary to nebulized agents. The prevalence of lesions suggest that VX, when administered by inhalation at concentrations of 171 and 343 mg × min/m^3, can induce mild but persistent pulmonary changes over several days postexposure. Wright et al. (2006) showed similar patterns in the lungs of guinea pigs microinstilled with VX for 5 min.

34.4.6 Cyanides

Cyanides were used as CWAs and are potential terrorist agents (Magnum and Skipper, 1942; NRC National Research Council, 1999). Cyanides are also used industrially for electroplating and the extraction of gold and silver. They can be released into the atmosphere from volcanoes, fungi, and bacteria. Common food items such as pears, peach, sweet potatoes, peas, apples, and lima beans are sources of cyanogenic compounds.

34.4.6.1 Exposure physiology

The cyanides, formerly known as "blood agents," consist of hydrogen cyanide (NATO code designation AC, HCN: MW 27.04) and cyanogen chloride (CK, ClCN: MW 61.5). The common metabolic by-product is the toxic CN^- anion, which is largely a systemic toxicant. Vapors from these agents are heavier than air and spread across the ground like phosgene and can pose a problem in low-lying, confined areas (Table 34.1). A mild exposure to these agents through the inhalation route can cause headache, loss of consciousness, ataxia, and confusion. Palpitations and respiratory tract irritation with labored breathing (dyspnea) can lead to hyperpnea in some cases.

For exposures classified as severe, serious CNS effects may occur. These include coma, seizures, and mydriasis. Dysrhythmias, low blood pressure, and eventual life-threatening cardiac arrest may follow. The occurrence of pulmonary edema followed by respiratory insufficiency can be a late manifestation following a severe inhalation exposure episode. The toxic load of inhaled HCN can be enhanced through its effect on increased minute volume (Purser et al., 1984).

Animal studies have provided evidence of the tissue distribution of inhaled cyanide. These studies have indicated that the target organs are the lung and heart, followed by the brain; all highly perfused. For example, in the rat following an acute inhalation exposure to HCN, the highest concentrations were measured in the blood, brain, heart, and lung with very little in the liver (Ballantyne, 1983). The estimated human inhaled 50% lethal concentration of HCN is 2500−5000 mg × min/m^3 and for cyanogen chloride, it is approximately 11,000 mg × min/m^3. These data indicate that via the inhalation route, the cyanides are less toxic compared to their effects as systemic poisons (McNamara, 1976).

34.4.6.2 Exposure biochemistry

It is well known that HCN vapor readily penetrates the epithelium with little difficulty due to its low ionization and low molecular weight. This allows for the rapid absorption through pulmonary alveolar membrane during exposure. The basic metabolic chemical reactions involving CN^- have been studied for decades (Sykes et al., 1981). CN^- binds to and inactivates enzymes involved in oxidative phosphorylation within mitochondria. The mitochondria are the primary target organelles involved in HCN poisoning. Lethal cytotoxic anoxia results from the interaction of CN^- with cytochrome c-oxidase, which is an important regulator of cellular respiration. This reaction, which occurs within minutes, inhibits aerobic metabolism by binding to the binuclear heme center. Additionally, there is loss of the formation of ATP, which is critical to supplying cells with the energy required for normal cellular processes (Keilin, 1929; Klein and Olsen, 1947). This adverse reaction prevents the binding of electrons to the molecular oxygen. As such, aerobic cell metabolism is halted even in the presence of well-oxygenated blood. In a futile attempt to regain the loss of ATP, cells increase glycolysis, especially in organs rich in mitochondria, such as the brain and heart. This results in metabolic acidosis characterized by increase in lactic acid. This, along with severely decreased ATP production, is responsible for dampened neurotransmission and altered perceptive abilities (Lindalh et al., 2004). In the brains of mice exposed to cyanides, lactic acid increases, and accumulation of adenosine diphosphate (ADP) and phosphates also lead to overall CNS metabolic impairment (Estler, 1965; Isom et al., 1975). CN^- can be removed through metal-complexing interactions with cobalt, molybdenum, and organic compounds prior to cell entry. Cyanide also forms cyanomethemoglobin as a result of its interaction with methemoglobin within erythrocytes. This process can remove cyanide from the plasma. Detoxification of CN^- occurs through the formation of the less acutely toxic thiocyanate (SCN) via the mitochondrial enzyme rhodanese which catalyzes the transfer of a sulfane sulfur atom from sulfur donors to sulfur acceptors (Eq. 34.2). The end reaction with the formation of SCN^- is generally irreversible.

$$CN^- + S_2O_3^{2-} \rightarrow SCN^- + SO_3^{2-} \qquad (34.2)$$

β-mercaptopyruvate-cyanide transulfurases, which are present in the liver, kidney, and blood are also capable of detoxifying cyanides forming SCN^- (Westley et al., 1981, 1983). A third enzymatic system present in the kidney may likewise play a role in the detoxification of CN^-. The sulfotransferase, called *cystathionine* γ-lyase, has been shown to be effective in cyanide-clearing processes (Wrobel et al., 2004).

34.4.6.3 Exposure histopathology

Inhalation exposure to cyanide-forming agents leave scant identifiable markers of tissue or cellular damage in target organs such as the lung, heart, and brain following challenge. This is mostly because once in the body, it diffuses rapidly into the bloodstream and acts essentially as a systemic toxicant, regardless of route of exposure. Several earlier animal studies showed that acute or repeated exposure to CN^- causes neuropathological changes, such as gray matter necrosis and degenerative changes in ganglion and Purkinje cells, in dogs (Haymaker et al., 1952). Encephalopathy and optic nerve neuropathy are other sites of toxicity following inhalational exposure (Hirano et al., 1967; Lessel, 1971). Thus, inhalational exposure might result in more severe neuronal impact, since the brain is rich in mitochondria.

34.4.7 Riot control agents

RCAs, also known as *sternutators* (from the Latin term meaning "sneezing") and *lacrimators*, consist of a specific class of irritant compounds. Agents (and NATO codes) in this class are 2-chlorobenzylidene malononitrile (CS: MW 188.6), dibenz (*b,f*)−1:4-oxazepine (CR: MW 195.3), oleoresin capsicum (OC: MW 305.4), 10-chloro-5,10-diphenylaminochlorarsine (DM: MW 277.6), chloropicrin (PS: MW 164.4), and 1-chlorocaetophenone (CN, also known as MACE: MW 154.6). Some of these agents, such as PS, DM, and CN, are CWAs (Fig. 34.2).

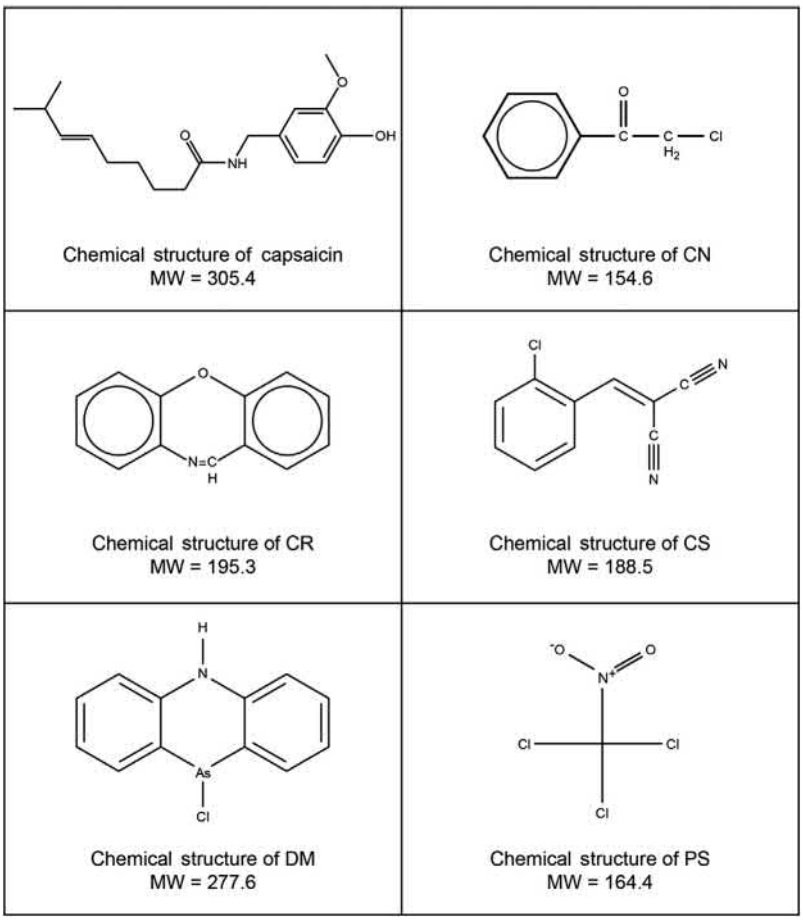


FIGURE 34.2 Chemical structures and formula weights of the most commonly studied RCAs.

RCAs have the general characteristics of causing rapid incapacitation, ease of dissemination, and relative low toxicity. They are usually disseminated as mists, aerosols, smoke, or volatilized M18 thermal grenades. They have been used widely as CWAs (Hilmas et al., 2008; Salem et al., 2008). CN was the original tear gas, which eventually was replaced by CS. In general, these agents possess actions that are short-lived, with the exposed becoming adapted to the effects within about 30 min of exposure. However, death has been known to occur with high doses and in confined spaces. Pharmacologically, they fall across classes that include emetics, hypnotics, serotonin antagonists, neuromuscular blockers, and sedatives. The toxicological effects of RCAs are readily apparent in various anatomical areas, such as the eye, gastrointestinal tract, nasal, oral and neuronal tissues, lung, and skin. Complex interactions among the sites described make RCAs potent candidates for crowd dispersion and control. Even though all of these anatomical sites are critical with regard to aftereffects, we will focus primarily on inhalation toxicology. For an outstanding and extensive review of RCA toxicity, see Olajos and Salem (2001).

34.4.7.1 2-Chlorobenzylidene malononitrile

CS is a white crystalline solid with a relatively low solubility in water, but a rapid rate of hydrolysis. It is soluble in most polar organic solvents. Harris (1993) describes RCAs as nonlethal, making them most effective as crowd dispersants on unprotected personnel. RCAs can enter the respiratory tract in the form of vapor or aerosol.

34.4.7.1.1 Exposure physiology

When inhaled, these agents are capable of sensory nerve receptor irritation, causing the Kratschmer reflex, which may result in cessation of respiratory function. Initial responses include sneezing, coughing, a burning sensation, and excessive rhinorrhea. Additional responses can also include dizziness and disorientation. While these are protective mechanisms to reduce the effects of inhalation, they may be accompanied by bradycardia and biphasic changes in aortic blood pressure. The half-life of CS following inhalation is less than 30 s (Olajos and Salem, 2001). Because of its low water solubility CS is environmentally persistent (Table 34.1). To further increase environmental persistence for up to 2 weeks, two hydrophobic

forms were synthesized, CS1 and CS2, which are rapidly absorbed from the respiratory tract. In rat inhalation studies, repeated exposure to CS1 caused aggressive behavior and hyperreactivity. In other studies with concentrations reaching 25,000–68,000 mg × min/m^3, there were no changes in blood electrolyte levels; however, pulmonary edema and necrosis of the respiratory and gastrointestinal tracts were observed in the rats that died after CS exposure. Death most likely consisted of the combined result of hypoxia, circulatory failure, and obstructed airways (Salem, et al., 2006). In human volunteers, exposures to a range of CS concentrations showed no evidence of deleterious changes in airway resistance, vital capacity, or tidal volume (Beswick et al., 1972). The LCt_{50} values for humans have been estimated to range between 25,000 and 100,000 mg × min/m^3 (WHO (World Health Organization), 1970). However, there have been no reported human mortalities due to CS exposure. The high levels of estimated concentrations of CS required to be lethal in humans clearly places it in the low lethality category (Table 34.1). While lethality is not measured during the course of human exposure, CS exposure has been linked to RADS, which can result from a single intentional exposure (Hu and Christiani, 1992). The respiratory irritation effects of CS exposure may be linked to the release of bradykinin (McNamara et al., 1969; Cucinell et al., 1971).

34.4.7.1.2 Exposure biochemistry

As noted previously, CS is rapidly absorbed during inhalation. It has a very short half-life and is metabolized and detoxified in the blood and other organs. Its disappearance follows first-order kinetics, and it spontaneously hydrolyzes to form malononitrile, which is then metabolized to thiocyanide (Nash et al., 1950). However, it is believed that in the case of inhalation exposure, the amount of formed thiocyanide may have little if any role in human toxicity (Ballantyne, 2006). Metabolites can be found in the blood only after high exposures. CS is an SN_2-alkylating agent with active halogens, which react with nucleophilic sites. A common metabolite 2-chlorobenzaldehyde was found in the blood of rodents and humans after exposure. CS reacts rapidly with plasma proteins and GSH, all of which are possible cofactors responsible for detoxification (Cucinell et al., 1971). Sulfhydryl-containing enzymes, such as lactic dehydrogenase, pyruvic decarboxylase, and glutamic dehydrogenase, are targets of the alkylating effects of CS (Lovre and Cucinell, 1970). The i.p. administration of CS to rats results in its excretion in the urine as mercapturic acid (Rietveld et al., 1983). It is not known if this occurs following inhalation. On the other hand, it has been reported that aerosol exposure to CS leads to an excretion of thiocyanate. The release of cyanide has also been shown to occur following i.p. injection in the rat (Frankenberg and Sorbo, 1973).

34.4.7.1.3 Exposure histopathology

Overall, CS does not produce significant respiratory tract or systemic lesions. In mice and rats, exposure to CS caused squamous metaplasia of the olfactory epithelium and hyperplasia and metaplasia in the respiratory epithelium. In 1990, the National Toxicology Program (NTP) tested CS for genotoxicity and carcinogenicity and found no evidence of either effect in rodents (NIH (National Toxicology Program), 1990). In inhalation experiments using rats and hamsters, with CS concentrations ranging between 150 and 750 mg/m^3 and lasting from 30 to 120 min, a small degree of lung pathology was seen for up to 28 days postexposure. The hallmark of exposure at high levels in the lung was congested alveolar capillaries, pulmonary edema, and hemorrhagic lesions. Secondary effects in extrapulmonary organs were renal tubular and hepatic cellular necrosis (Salem et al., 2006). In 120-day inhalation studies using guinea pigs, acute alveolitis was observed (Ballantyne and Callaway, 1972; Ballantyne and Swanston, 1978).

34.4.7.2 Dibenz (b,f) − 1:4-oxazepine (CR)

CR is a pale yellow solid with a strong, pepperlike odor. Exposure-effect responses of CR are similar to CS. CR is the most potent lacrimator with the least toxicity. It is the parent compound of the antipsychotic drug loxapine (Blaine, 2003).

34.4.7.2.1 Exposure physiology

Upon inhalation, CR is rapidly absorbed through the lung and into the bloodstream. It has a plasma half-life of about 5 min (Upshall et al., 1977). CR can be dispersed as an aerosol or as grenade-generated smoke. For the most part, animals exposed to CR exhibit physical dysfunctions such as ataxia, loss of coordination, tachypnea, and convulsions. In human volunteers, low-level CR aerosol exposure produced bronchoconstriction and increased pulmonary blood flow within about 20 min (Ashton et al., 1978). These effects are believed to be due to the stimulation of irritant receptors, the effects of which usually subside within an hour after exposure. Ballantyne studied the effects of inhaled CR aerosol and CR smoke in rats. Concentrations ranged between 13,050 and 428,000 mg × min/m^3. Excessive nasal discharge and blepharospasms occurred, which also subsided in about 60 min (Ballantyne, 1977). No mortalities were recorded. Investigations involving the effects of CR exposure on teratogenicity (specifically, embryo toxicity) revealed no adverse developmental toxicology (Upshall, 1974).

Carcinogenicity and genotoxicity assessment using various strains of *Salmonella typhimurium* reverse mutation tests, CHO gene mutation system (V79/HGPRT), mouse lymphoma assay (L5178Y/TK + /TK −), and micronucleus testing also indicated no toxicity (Colgrave et al., 1979). Data from Kumar et al. (1995, 2006) provides evidence that CN is much more toxic than CR at the 5% LC_{50} level and upon repeated exposure in rats and mice.

34.4.7.2.2 Exposure biochemistry

In rat tissue, CR is metabolized to 4-, 7-, and 9-hydroxylactams via oxidative processes, followed by ring hydroxylation and sulfate conjugation, and then excreted via urine (French et al., 1983). Metabolites and excretion profiles of CR are similar among many species regardless of exposure route. Intravascularly dosed mice suggested rapid uptake of CR from the blood and into the kidney, small intestine, bile, and liver, which was in line with data from rats indicating rapid absorption, hepatic metabolism, and renal excretion (French et al., 1983).

34.4.7.2.3 Exposure histopathology

CR administered by aerosol inhalation at 68,400 mg × min/m^3 did not cause lethality in mice, rabbits, or guinea pigs (Ballantyne, 1977). However, congestion in the capillaries and alveolar hemorrhaging were noted. Pattle et al. (1974) exposed rats to CR at a concentration of 115,000 mg × min/m^3 to determine whether a high concentration produced adverse effects on cell organelles such as lamellar bodies. Electron microscopy indicated no effects of inhaled CR on lamellar bodies. Colgrave et al. (1979) exposed animals to a range of CR aerosols at dosages from 78,200 to 161,300 mg × min/m^3 and reported no abnormalities in the lung. However, subsequent assessment after microscopic examination revealed mild lung congestion, hemorrhage, and emphysema. Long-term effects of inhaled CR aerosol were conducted in hamsters and mice using dosages of 204 ppm (5 min), 236 ppm (8.6 min), and 267 ppm (15.8 min) 5 days/week for 18 weeks. Survivors were sacrificed and dissected 1 year after exposure, with the only pathology being chronic laryngeal inflammation (Marrs et al., 1983).

34.4.7.3 10-Chloro-5,10-diphenylaminochlorarsine (DM-adamsite)

DM, an organoarsenical compound, is a yellowish-green crystalline solid with low volatility. DM and other compounds, such as PS, diphenylchloroarsine (DA), and diphenylcyanoarsine (DC), are known as *vomiting agents*. DM is considered odorless, but it can contain a faint odor of bitter almonds. It is more soluble in organic solvents, such as benzene, toluene, and alcohols, than in water. It can be released as a dry powder using thermal or explosive techniques.

34.4.7.3.1 Exposure physiology

Respiratory effects of inhaled DM include increased nasal congestion, salivation, coughing, and sneezing. Irritation of the mucous membranes of the eyes and airway lining can occur. Persistent vomiting and headaches are also exhibited after DM exposure. Compared to other RCAs, the effects of inhaled DM can take as long as 3 min and last up to several hours (BMOD British Ministry of Defense, 1972). Punte et al. (1962) studied the effects of a number of RCAs in animals that were exposed for periods ranging from 5 to 90 min. With DM, there were no abnormalities observed below concentrations of 500 mg × min/m^3. Estimated LCt_{50} values were as follows: for mouse, 22,400 mg × min/m^3; rat, 3700 mg × min/m^3; and guinea pig, 7900 mg × min/m^3.

Striker et al. (1967) exposed nonhuman primates to various $[C] \times t$ levels of DM. An inhaled dose of 2565 mg × min/m^3 was well tolerated except for one animal that exhibited nasal discharge. At a dose level of $[C] \times t$ of 8540 mg × min/m^3, nasal discharge and facial erythema were noted, but these symptoms were resolved by 24 h post-exposure. When the exposure dose was increased to 28,765 mg × min/m^3, hyperactivity ensued, with significant nasal discharge and respiratory distress in all animals. Striker et al. (1967) concluded that death in some animals was caused by respiratory failure. In human volunteers, doses thought to cause adverse physiological responses such as nausea and vomiting were tested. Some studies found that humans can tolerate DM in the range of 22−92 mg/m^3 for at least 1 min with a 50% tolerance level of up to 220 mg/m^3 (Punte et al., 1962). In this study, the definition of tolerability was the desire to vacate the exposure space as quickly as possible. From these studies and others, it was shown that exposure to inhaled DM produced a range of effects including pain in the eyes, throat, nose, and upper airway; coughing; and salivation similar to a cholinergic response seen with nerve agent exposure. If the exposure is severe enough and sufficient in duration, death may ensue. There is one known human death caused by inhaled DM (Owens et al., 1967). Exposure times of 5−30 min at concentrations of 1130−2260 mg/m^3 ($[C] \times t$ of 5650 to 67,800 mg × min/m^3) were estimated. For the most part, this is well above the estimated LCt_{50} of 11,000 mg × min/m^3 for humans reported by Sidell et al. (1997a,b).

34.4.7.3.2 Exposure biochemistry

There is only one study that suggests the possible metabolic and detoxification fate of DM (Haas et al., 2004). As an organoarsenical compound, it would seem likely that its toxicity may be linked to the metabolism of arsenic. Through the oxidation of As (III) by manganese peroxide, DM is broken down into As (V). This reaction releases chloride with the subsequent incorporation of two oxygen molecules into the parent compound (Haas et al., 2004).

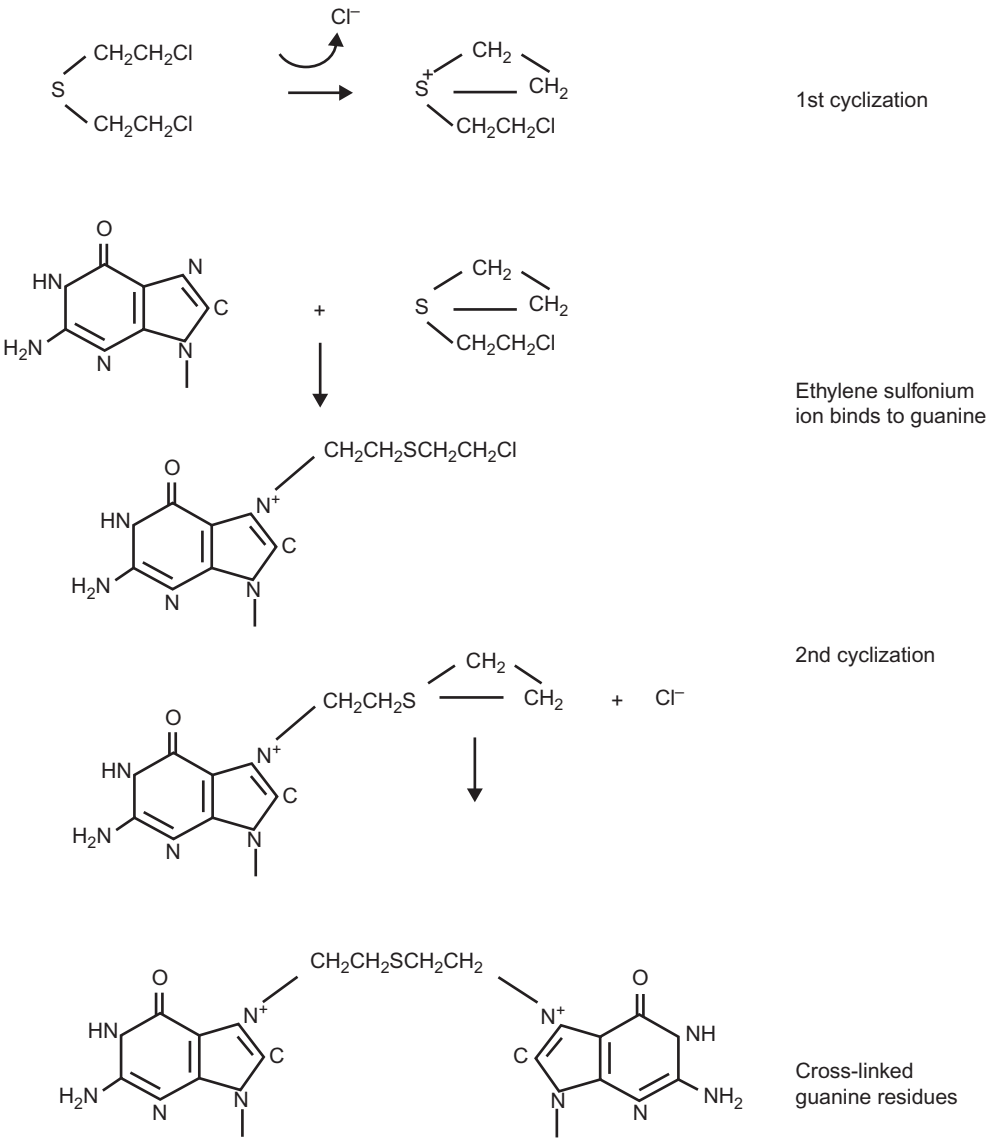


FIGURE 34.3 Reactive chemical mechanisms involved in HD hydrolysis, formation of sulfonium ion, and the alkylation of DNA through guanine cross-linked residues.

Arsenic released in the metabolic process with a specific valance state could inhibit succinic dehydrogenase activity and cause the uncoupling of oxidative phosphorylation and reduced ATP levels.

34.4.7.3.3 Exposure histopathology

As stated previously, there remains little doubt that the target organ showing significant and consistent debilitating effects of inhaled DM is the lung. In DM-exposed nonhuman primates, gross postmortem examination showed evidence of edematous and severely congested lungs, whereas microscopic evaluation revealed ulceration of the tracheobronchial tree and alveolar edema (Striker et al., 1967). In the human death mentioned previously, postmortem analysis revealed edema of the subcutaneous tissue of the neck, mediastinum, pleura, and pericardium, along with emphysematous bullae widespread in the lung. Histologically, bronchopneumonia, hemorrhage, pseudomembrane formation in the trachea and bronchi, and congestion were observed throughout the entire respiratory tract (Owens et al., 1967).

34.4.7.4 Oleoresin of capsicum (OC—pepper spray)

OC is a reddish-brown, oily resin derived from capsicum plants, commonly known as nightshade (Fig. 34.3). Capsaicinoids can exist in several derivative forms such as dihydrocapsaicin, homocapsaicin, and nordihydrocapsaicins. Capsaicinoids are isolated through volatile extraction of the dried, ripened fruit of chili peppers. Once dried, the OCs are predominantly capsaicin, and also nordihydrocapsaicin and homocapsaicin (Salem et al., 2006).

However, other potentially irritating components are present, such as acids, esters, and phenolic agents. Capsaicins at high concentrations can cause a variety of potent effects such as dermatitis, nasal, ocular, pulmonary, and gastrointestinal tissue injury. The active ingredient of capsaicin is 8-methyl-*N*-vanillyl 1,6, nonenamide.

34.4.7.4.1 Exposure physiology

Like all other RCAs, aerosol-dispersed OC causes erythema; burning of the eyes, nose, and throat; sneezing; coughing; and blepharospasm. Acute exposure to inhaled OCs results in pulmonary edema, bronchospasm, respiratory arrest, hypertensive crises, and hyperthermia. OC causes excitement, convulsions, dyspnea, and death due to respiratory failure. While pepper spray is considered relatively safe, fatalities have been reported due to airway obstruction (Synman et al., 2001). Inhalation of capsaicin activates the Kratschmer reflex, as mentioned earlier, accompanied by bradycardia, apnea, and a biphasic rise and fall of aortic blood pressure. The pulmonary effects of capsaicin may be species-related. In guinea pigs exposed to capsaicin via aerosol, bronchoconstriction occurred, suggesting both a vagal-cholinergic as well as a noncholinergic local axonal reflex (Buchan and Adcock, 1992), whereas in cats using a similar exposure method, bronchoconstriction was determined to be due to a direct vagal-cholinergic response (Adcock and Smith, 1989). Glinsukon et al. (1980) determined the LD_{50} in several animal species using various routes of delivery. The concentrations ranged from 0.56 mg/kg intravenously (i.v.) to 1.6 mg/kg by intratracheal instillation. An LCt_{50} value for capsaicin of 13,000 mg/m^3 × min has been published by Seto (2011).

34.4.7.4.2 Exposure biochemistry

One of the mechanisms responsible for capsaicin toxicity is the stimulation of neurons consisting of neuropeptide-containing afferent nerves that are capable of activating specific vanilloid receptors (Szallasi and Blumberg, 1999). Stimulated nerves are of the Aδ and C fiber-unmyelinated types. The activation through ligand-gated mechanisms opens cation channels (Marsh et al., 1987) and increases the influx of Ca^{2+} and Na^{+} in cells, which leads to depolarization and the release of neuropeptides such as substance P. Protective reflexes are activated via autonomic motor neurons. Other transmitters of biological responses to capsaicin included calcitonin gene-related peptide (CGRP) and neurokinin A (a tachykinin), both of which are released from neurons and are responsible for pain transmission and neurogenic inflammation. For an extensive list of capsaicin-sensitive markers, see Holzer (1991).

Mediator-induced inflammatory processes can lead to increased vascular permeability, neurogenic inflammation of airways and blood vessels, chemotaxis, and bronchospasm (Smith and Topford, 1999). Furthermore, capsaicin activates the vanilloid receptor family TRPV1 (transient receptor potential cation channel subfamily V) within sensory neurons. Activation of these receptors leads to prolonged refractory periods and to a nonconducting desensitization. One study suggests that TRPV pathways may not be entirely responsible for all of the adverse reactions caused by capsaicin (Holzer, 1991).

34.4.7.4.3 Exposure histopathology

There have been no reports or reviews assessing the histopathology results from an inhalation challenge to capsaicins or OCs. The only data from toxicity studies was from Glinsukon et al. (1980), who found visceral organ hyperemia without hemorrhage, gastric desquamation, and increased mucus.

34.4.7.5 Chloropicrin (PS)

PS produces many of the effects consistent with RCAs. In addition, it has been classed with choking agents such as phosgene and chlorine due to its effect on the upper airways. PS is an oil ranging from colorless to light green, with a stinging odor classified as aniselike. It has low solubility in water, but it is readily soluble in most solvents such as acetone and chloroform. At high temperatures, PS decomposes to phosgene, nitrogen dioxide, and other irritants. During World War I, it was labeled as a choking agent and mixed with sulfur mustard to lower its freezing point. PS is a lung irritant and is used as a soil fumigant for its biocidal and fungicidal properties. As a result of commercial usage, exposure threats are by way of the inhalation and dermal routes in persons living and working within the operational area (O'Malley et al., 2004).

34.4.7.5.1 Exposure physiology

Mechanisms of PS toxicity are not that well understood. Largely, PS is irritating to the respiratory system, mucous membrane, gastrointestinal tract, skin, and eyes. Airway inflammation, lacrimation, nausea, and vomiting are the hallmarks of exposure. In severe respiratory exposures, pulmonary edema can ensue, ultimately causing death. In mice inhalation studies, Buckley et al. (1984) assessed the RD_{50} (the dose of material required to cause a 50% reduction in respiration rate), which was 8 ppm with exposures of 6 h/day over 5 days. Exfoliation, ulceration, and necrosis of the respiratory epithelium were observed. The estimated LCt_{50} is 2000 mg × min/m^3. A 20-min exposure to PS in pigs and cats resulted in an LD_{50} value of 800 mg/m^3 or 16,000 mg × min/m^3, respectively; and for mice, 15,840 mg × min/m^3 over a 4-h exposure (NIOSH

(National Institutes of Occupational Safety and Health), 2007). For humans, the permissible exposure limit (PEL) established by the National Institute of Occupational Safety and Health (NIOSH)/Occupational Safety and Health Administration (OSHA) for a work-related, time-weighted average is 0.7 mg/m^3 (1 ppm = 6.72 mg/m^3). With regard to the capacity of PS to be genotoxic, carcinogenic, or both, studies have shown mixed results.

34.4.7.5.2 Exposure biochemistry

As with several RCAs, the absorption, distribution, metabolism, and excretion of PS are not well elucidated. However, in two studies by Sparks et al. (1997, 2000), data indicated that PS can form adducts with thiol groups. After oral or i.p. injection in mice, PS is rapidly absorbed and distributed to the blood, liver, and kidneys. Metabolites identified include thiophosgene dichloronitromethane, chloronitromethane, and nitromethane. Castro et al. (1988) and Sparks et al. (1997, 2000) both suggest that enzymatic reactions may be driven by β-lyase, cytochrome P-450s, and GSH-S-transferases. Additional evidence suggests that PS may be converted to raphanusamic acid (Sparks et al., 1997).

34.4.7.5.3 Exposure histopathology

In postmortem assessment of exposure to inhaled PS, Buckley et al. (1984), recorded that PS was only one of several inhalants that produced lower respiratory tract lesions. In a case of homicidal poisoning resulting in an exposure to PS, spotty discoloration and pulmonary edema were found in the postmortem examination (Gonmori et al., 1987). Death in this case occurred within 3−4 h of exposure.

34.4.7.6 1-Chloroacetophenone (CN)

CN is a gray, crystalline solid compound with the odor of apple blossoms. Known also as Mace, it was specifically developed as an RCA. A potent lacrimator, it was developed just after World War I. As a vapor, it is 5 times heavier than air. Dissemination of CN can be achieved by grenade, steam dispensers, projectile cartridges, and aerosols.

34.4.7.6.1 Exposure physiology

CN is a much more effective lacrimator than either CS or CR, causing serious injury to the ocular targets. Blepharospasm, corneal edema, erosion, and ulceration, as well as focal hemorrhages, can occur. As a potent skin toxicant it produces severe skin edema. Irritation of the airway, including sneezing, coughing, nasal secretions, and congestion, may persist for up to 20 min. after a challenge. The primary cause of death is from damage to the lung.

34.4.7.6.2 Exposure biochemistry

The LCt_{50} for several species using the aerosol delivery method are as follows: dog, 7033 mg × min/m^3; rat, 8878 mg × min/m^3; and guinea pig, 7984 mg × min/m^3. Multiple exposures using nonhuman primates and guinea pigs demonstrated that lethality decreases with repeated exposures over days or weeks, suggesting adaptation to CN toxicity. Using guinea pigs, dogs, and nonhuman primates in a similar experimental design, with a 10-day concentration totaling 88,000 mg × min/m^3, mortality was observed in animals. In one of the few comparative inhalation studies, Ballantyne and Swanston (1978) performed acute toxicity testing on rats, mice, guinea pigs, and rabbits using CN and CS aerosols. The duration of exposure ranged from 15 to 60 min, with lethality occurring within 14 days. The respective LCt_{50} values for these animals were 8750, 18,200, 13,140, and 11,480 mg × min/m^3. For all species, CN was much more toxic than CS. Equitoxic doses of CN and CS produced similar findings using the i.v. and i.p. challenge routes. These data suggests that for appropriate toxicity screening, multispecies and multiagent comparisons are critical. For further toxicological reviews, see McNamara et al. (1969).

CN has been shown to increase lung sphingomyelin and decrease phosphatidylcholine and ethanolamine in rats (Kumar et al., 1995). Based on the increased bacterial retention in the lung in mice exposed to CN, the authors concluded that CN might increase susceptibility to infection. This may be attributed to the immunotoxic effects of CN through altered, T-cell-mediated macrophage functions (Kumar et al., 1992).

34.4.7.6.3 Exposure histopathology

Animals exposed to high concentrations of CN that resulted in mortality at 48 h showed evidence of tracheal congestion and pulmonary edema (Ballantyne and Swanston, 1978). Histopathology revealed congestion of alveolar capillaries and intrapulmonary veins, inter- and intra-alveolar hemorrhages, and excessive secretions in the bronchioles. CN was reported to produce degeneration in the epithelium of the bronchiole and alveolar septal-wall thickening and the presence of an increased number of mononuclear cells in rats (Kumar et al., 1995). Necrosis of the liver, kidney, small intestine, and spleen were also observed.

34.4.8 DA and DC

As previously stated, DA and DC are known as *vomiting agents*. Like other RCAs, they manifest their toxicity by

irritating the airway mucous membranes, as well as the eyes. Irritation results in sneezing, coughing, vomiting, and headache. They have a bond between trivalent arsenic and chlorine (in the case of DA) or cyanide (in the case of DC) and are less toxic than CN.

DA, a sternutator, is a white crystalline solid with a MW of 265. As was noted for DM, the toxicity of these compounds is attributable to the presence of arsenic in the form of As (III). The general, physiological outcome of exposure to organoarsenicals can be manifested as severe conjunctival membrane irritation, swelling of the cornea, and sloughing of respiratory mucosa forming mucus plugs in the lower airways. DA can produce these effects, and it leads to death if exposure occurs in unventilated and confined spaces (Ochi et al., 2004). Studies by Ishii et al. (2004) postulated that metabolic by-products of DA and DC, such as diphenylarsenic acid, might be associated with developmental abnormalities in humans. The estimated LCt_{50} values for DA and DC are 15,000 and 10,000 mg $\times$ min/m^3, respectively (Seto, 2011). It should be noted, however, that the toxicity of DA as a CWA is lower than the vesicants discussed next. Limited information is available on the pulmonary toxicity of these agents.

34.4.9 Vesicating agents

Sulfur mustard was used extensively in World War I and was responsible for thousands of casualties. The most recent significant intentional use of vesicants was that of sulfur mustard (HD) during the Iran–Iraq war (1984–88). Vesicants are compounds that produce chemical burns. Members of this group of vesicating agents include HD, lewisite (also known as *Agent L*) (an arsenical) and phosgene oxime (CG). CG exposure produces hivelike, urticarial skin reactions on contact. HD is a persistent agent as it can remain on the soil for more than a day (Table 34.1). Persistence is dependent on pH, soil type, and hydrophilicity. At high temperatures, such as those that occur in the desert (38–49°C), it is a considerable inhalation hazard. There is a great deal of literature regarding the toxicology, physiology and biochemistry of HD.

34.4.9.1 Sulfur mustard—bis-(2-chloroethyl) sulfide (HD)

Sulfur mustard, or mustard gas, is a colorless oil with a MW of 159. Impurities can cause HD to give off a garlic-like odor. It is 5.4 times as dense as air, which enables it to settle in trenches (and hence making it well suited for war). Although it is a low volatility agent, the HD vapor was responsible for about 80% of the chemical exposure deaths during World War I. HD has also been known as "Yellow Cross," "Lost," "S-Lost," "H," or "Yperite." Because HD is denser than water and does not readily hydrolyze, it can remain a threat for a while in various aqueous environments and has caused skin blisters when handled many years after submergence (Aasted et al., 1985; Wulf et al., 1985). The formation of fluid-filled blisters by HD can cause immediate tissue damage yet latent pain that may take several hours. Clinical effects may occur hours later.

34.4.9.1.1 Exposure physiology

Although skin is considered to be an important route of exposure for HD, many of the fatalities ascribed to HD exposure during World War I and the Iran–Iraq war were the result of inhalation. Inhaled HD is largely absorbed in the upper airway and rarely penetrates to the lung parenchyma unless the concentration or duration of exposure are sufficiently high. In humans, following inhalation exposure to HD, reports have identified bronchopneumonia, chest tightness, and, in long-term survivors, chronic bronchitis, lung fibrosis, productive cough, and chronic obstructive pulmonary disease (Ghanei and Harandi, 2007). Injury can develop slowly, becoming much more intense over time. One important issue with HD-induced lung damage is the proper maintenance of blood oxygenation. Hypoxia is usually secondary to inflammation and bronchitis. Other consequences following exposure include tracheobronchial stenosis and life-threatening pseudomembrane (casts) formation. Leukopenia can also occur several days after exposure and suggests compromise of the immune pathway that can progress to suppressed bacterial clearance and sepsis. HD exposure has also been shown to affect the CNS. Most of the casualties observed following an inhalation exposure to HD were from pulmonary damage generally due to complications of bronchopneumonia, sepsis, and immunosuppression. Experiments done in animals decades ago employed various routes of administration, including inhalation, which produced convulsions, hyperactivity, and abnormal movements (Vedder and Vedder, 1925). Anderson et al. (1996) demonstrated the formation of mucus casts within 24 h of exposure in a HD vapor rat inhalation model. Casts can cause ventilation perfusion mismatch due to clogged airspaces and lead to respiratory failure. Heully and Gruninger (1956) reported that three children accidently exposed to HD had abnormal muscular activity with one child alternating between coma and agitation. Two of these children died.

In large animal studies, such as with swine, 6 h after a 10-min exposure to HD vapor from 67 to 157 μg/kg body weight resulted in respiratory acidosis, increased shunt fraction, and hypoxemia at the higher concentrations. Elevated bronchoalveolar lavage (BAL) levels of IL-8 and IL-1β were measured in the high-dose exposed

animals suggesting a pro-inflammatory stimulation associated with airway injury (Fairhall et al., 2010). The LCt_{50} of HD has been estimated to be 1500 mg × min/m^3 (Seto, 2011).

34.4.9.1.2 Exposure biochemistry

The mechanism of biotransformation of HD has been elucidated. Once HD enters the target organ and blood, it forms an intermediate sulfonium ion which is transformed into a carbonium ion considered to be a strong electrophilic species capable of reacting with highly nucleophilic sites (Fig. 34.3). These are robust alkylating agents that react with cellular constituents, such as SH^- and NH^- in DNA, RNA, lipids, and proteins. Byrne and Stites (1995) have shown that HD forms crosslinks with cysteine residues in proteins. HD reacts with RNA and DNA molecules at such sites as N7 guanine, N3 adenine, and O^6-guanine. The O^6 position is particularly critical for the action of O^6-alkyl-guanine-DNA-alkyltransferase in DNA repair (Ludlum et al., 1986) (Fig. 34.3). HD also forms DNA crosslinks. Kehe and Szinicz (2005) have discussed the likely role of HD in alkylating membrane-bound proteins and enzymes. Zhang et al. (1995) have assessed the effects of HD on basement membrane components. Their results indicated that HD can alkylate and form crosslinks with membrane adhesion molecules such as laminin. This may have important consequences in the case of a high-dose inhalation exposure to HD, whereby it can reach and directly affect the alveolar epithelial cell layer. Bone marrow, a site of cell proliferation, is also a target of HD toxicity (Dacre and Goldman, 1996). In 2008, Kan et al. provided some evidence that there may be causal link between the inhalation of HD and cancer in rats exposed to a single high dose of HD (3 mg total dose over 10 min) (Kan et al., 2008). The conclusions of this limited study have yet to be supported by continued research. However, the results obtained may provide the incentive for future studies.

In rodent studies using intravascularly administered radiolabeled HD, distribution occurred in highly perfused organs such as the kidney, heart, liver, intestine, and lung within 1 h after injection (Maisonneuve et al., 1994). These organs are sites of biotransformation. Capacio et al. (2008) showed that HD-plasma protein adducts can be measured in the blood following a single 10-min exposure to graded increases of HD in a rat aerosol inhalation model.

The role of GSH in detoxification of HD has been somewhat controversial. Sciuto et al. (2007) have shown that there may be a biphasic response to HD intoxication. Testing for pulmonary toxicity using an intravascular HD injection model in rats, it was shown that at 1 h postexposure, BAL revealed a dose-dependent inflammatory stage that decreased over time from 3 to 24 h. Macrophage inflammatory protein (MIP-2) was the predominant chemokine in the BAL followed by IL1β, IL10, and TNFα. A second phase involved changes in the antioxidant response pathway, which was mostly affected at 6 h post-challenge. HD exposure increased BALF protein, GSH, and the activities of GSH peroxidase (GPX), catalase, GSH reductase, and SOD (Sciuto et al., 2007). Laskin et al. (2010) summarized that in some animal models of acute lung injury with HD and HD-like compounds, antioxidant markers such as GPX and GSH increased, suggesting enhanced endogenous compensatory antioxidant activity. Brimfield et al. (2012) investigated mustard-induced, oxygen free-radical formation in an *in vitro* system using the spin-trapping technique. They concluded that the presence of peroxyl and hydroxyl free radicals could arise only when oxygen is reduced by carbon-based mustard radicals. The electron paramagnetic resonance (EPR) ROS signals disappeared by adding catalase and SOD to the reaction system.

Increased autophagy has also been reported in rats exposed to HD. Increases in LC3B-II and LC3B I were noted in rat lungs as early as 6 h and as late as 48 h post-exposure (Malaviya et al., 2010). Since this process might involve mitochondria, it is possible that HD might induce alterations in cell energy and metabolism (Uchiyama et al., 2008, from Malaviya et al., 2010). Dillman et al. (2005) showed that apoptotic pathways are also activated within 3 h of intravascular injection of HD in rats. This damage subsequently resulted in enhanced expression of proteins that arrest cell cycle progression and enhance programmed cell death. Presumably, apoptotic activation in the face of extensive DNA damage may be intended to avert multiple mutations that could lead to cancer formation. It is also apparent that tissue and cell damage is dose-related and does not appear to decrease over time, suggesting a long-term effect in the lung from a single HD challenge.

It is apparent from battle casualties (most recently from the Iran−Iraq war) that both soldiers and civilians continue to suffer debilitating compromised lung function 30 + years after initial exposure (Ghanei and Harandi, 2007). However, the long-term consequences of HD inhalation exposure have not been well examined in animals. Mortality at 3−8 and 15−19 weeks following a single HD intravenous exposure (3 mg over 10 min) occurred in rats (Fig. 34.4). This dose approximately translates to LCt_{70} and LCt_{80}, respectively, over these time intervals. The data indicated that rats surviving a first phase with respect to lethality are not completely free of problems later. These data are supported by depressed respiratory function, which also suggests a link with temporal morbidity outcomes. Results from this long-term study in HD-exposed rats also revealed compromised respiratory function and symptoms of chronic

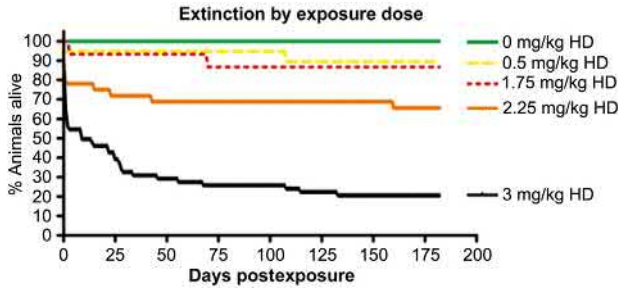


FIGURE 34.4 Shown here are 26-week survival curves of male rats exposed to a single 10 min inhaled HD. Concentrations represent the total dose delivered during exposure. Data indicate a delay in lethality based on dose. Exposures were done via intratracheal administration of aerosol in ventilated and anesthetized animals to avoid scrubbing in the nasal passages and providing a direct challenge to the lung. To control the rate of aerosol delivery, ventilation parameters (such as the tidal volume and the respiratory rate) were held constant for all exposed animals. *Data from Olivera, D., Sciuto, A.M. Unpublished research results.*

obstructive pulmonary disease in rats that are similar to those described in soldiers and civilians exposed to HD during the Iran−Iraq war.

Biomarkers of HD exposure have focused on blood and urine evaluations. Historically, the metabolite thiodiglycol (TDG) was used as an indicator of exposure. However, the significance of linking TDG to HD exposure became problematic since TDG levels in the urine can be naturally elevated as a result of normal metabolism. However, as analytical techniques improved, more refined analyses revealed other metabolites that were indicative of actual HD exposure. In a large swine HD inhalation study, the monosulfoxide β-lyase metabolite of HD [1-methylsulfinyl-2-[2(methylthio) (ethylsulfonyl) ethane] (MSMTESE)] was discovered in the urine (Fairhall et al., 2010). MSMTESE was present for up to 6 h postexposure following a single 10-min inhalation challenge to 158 μg/kg HD. The detection of MSMTESE in the urine served as a valid biomarker for an HD inhalation exposure in this model.

34.4.9.1.3 Exposure histopathology

In 15-year survivors of the Iran−Iraq war, histopathological examination of lung tissues revealed evidence of increased asthma, chronic bronchitis, emphysema, airway stenosis, bronchiectasis, and lung cancer, and possibly lung fibrosis as well. The contributions of smoking and the presence of preexisting pulmonary disease as potential confounders against these conclusions were not taken into account.

34.4.9.2 Lewisite—b-chlorovinyldichloroarsine (agent L)

Lewisite (also called *Agent L;* MW = 207.3) is a colorless, oily liquid. Impure Agent L may have a faint odor of geraniums and could be blue-black in color. It is classified as a blistering, but not vesicating, agent due to its effects on the skin and eyes. Compared to HD, Agent L forms fluid-filled blisters accompanied by immediate pain. Exposure occurs through inhalation, ocular, or dermal routes. After acute dermal exposure, the liver, gallbladder, urinary bladder, lung, and kidneys are affected as well (Snider et al., 1990). The appearance of blisters can begin at any time, ranging from seconds to minutes based on exposure $[C] \times t$. Lesions resulting from various exposure routes are similar to HD. Hydrolysis products are more persistent than HD, and it is about 10 times as volatile. It is soluble in organic solvents and is readily absorbed by paint, rubber, and porous surfaces. As an organoarsenical, the enhanced reactivity of Agent L is linked to labile chlorine atoms, trivalent arsenic, and its capacity to form multiple chemical bonds with proteins. Agent L contains about 0.8−1.3 mg/mL of arsenic.

34.4.9.2.1 Exposure physiology

Exposure to Agent L can occur through oral, dermal, ocular, ingestion, and inhalation pathways. Lewisite shock is observed following exposures to high levels. This mostly results from protein and plasma leakage from the capillaries, hypotension, and hemoconcentration. Agent L can readily penetrate the skin in both vapor and liquid form, causing systemic exposure; as little as 0.5 mL can cause serious problems, and at 2 mL, lethality can occur in humans. Vapor inhalation causes rhinorrhea, violent sneezing, and irritation to the nasal passages. These may be followed by expectoration of blood and coughing. According to Sidell et al. (1997a,b), Agent L does not cause bone marrow damage or immunosuppression. Clinically, large doses of arsenical compounds may lead to leukopenia. Since trivalent arsenic, As (III), is a major metabolite of Agent L, acute exposures might cause neurological damage. These may include loss of memory, problems in concentrating, anxiety, and confusion (Rodriguez et al., 2003). The inhaled LCt_{50} is estimated to be 1500 mg × min/m^3 in mammalian species (Seto, 2011). These are close to earlier estimates of inhalation studies in mice, where the LCt_{50} for a 10-min exposure was 1900−2000 mg × min/m^3 (Silver and McGrath, 1943).

Currently, there is insufficient data on the carcinogenic effects of Agent L. Combustion by-products of Agent L, such as arsenic trichloride, vinyl chloride, and arsenic trioxide, have the potential to induce cancer. Arsenic trioxide and vinyl chloride are labeled Class A carcinogens by the U.S. Environmental Protection Agency (US EPA US Environmental Protection Agency, 1984). However, it is not known if Agent L has any direct effect on cancer development. See Goldman and Dacre (1989) for a more detailed review on the chemistry of lewisite toxicity.

34.4.9.2.2 Exposure biochemistry

Agent L will react with water to form chlorovinylarsenous acid (CVAA), which is slowly converted to arsinoxide. Metabolites can be found in the urine after exposure (Waters and Williams, 1950). It is highly distributed systemically, even after inhalation, due to its high lipophilicity. The reaction by-products include unstable chlorine atoms, As (III), and carbon. It is most likely that the metabolite As (III) is responsible for the observed systemic toxicity. As (III) can react as a nucleophile on thiol groups in proteins and lipids, particularly on lipoic acid, eventually forming alkylarsine sulfides. The binding of Agent L to sulfhydryl-containing proteins and enzymes inhibit pyruvate dehydrogenase activity, which is critical to glucose and fatty acid metabolism (Black, 2008). Agent L metabolites produce antioxidant imbalance, calcium metabolism imbalance, lipid peroxidation associated with oxidative stress, membrane impairment, and ultimately cell death (Sidell et al., 1997a,b). Fidder et al. (2000) incubated labeled Agent L with human erythrocytes and found that 25%–50% of the bound isotope formed protein adducts at cysteine residues of 93 and 112 of the β-globin molecule (Noort et al., 2002). The major excretion by-products of Agent L, including CVAA, are quantifiable markers in the blood that can be useful in detecting exposure.

34.4.9.2.3 Exposure histopathology

There have been very limited studies on the toxicity of inhaled Agent L, specifically in the lungs. Decades ago, Vedder and Vedder (1925) showed that in dogs exposed to Agent L, considerable nasal secretions, lacrimation, vomiting, and labored breathing were present until death. At postmortem, lungs were found to be edematous, with pseudomembranes extending from the nasal area to the bronchi. Airway mucosa was congested and edematous. Bronchopneumonia was also present. No other studies have systematically examined pulmonary pathological changes in animals exposed to Agent L.

34.5 Concluding remarks and future directions

The potential for human exposure due to intentional use or accidental release of CWAs and toxic industrial chemicals will likely persist for many years. History has shown that exposure to these chemicals, regardless of the source, can be problematic for both civilians and military personnel. As such, continued study of the toxicity of these agents is of the utmost importance. All agents have the potential to cause significant morbidity and mortality by way of severely compromised pulmonary physiological homeostatic processes. The key target organ is the lung, and in many cases, regardless of the exposure route, respiratory failure is the cause of death. Subsequent exposure-related effects can involve altered respiratory function, compromised vital cellular metabolic/detoxification pathways, mitochondrial toxicity, hematotoxicity, and CNS toxicity. Other effects include immune response and associated release of cytokines and chemokines, altered antioxidant/oxidant pathways, increased free-radical formation, destruction of lung solute transport channels, and disruption of adaptive repair processes. All of the processes discussed here appear to be affected in a concentration-dependent manner; often with a latency period. For example, vesicant inhalation significantly alters the architecture of the lung to the extent that attempts to counteract the effects of damage through the innate repair process are compromised, leading to an obstructive pulmonary disorder.

The works cited herein show that although progress has been made in elucidating the toxicological, physiological, biochemical, and histopathological processes responsible for respiratory toxicity, much more effort is required to understand chemical-specific mechanisms and develop effective therapeutic measures. Three major issues will need to be addressed by future studies: (1) the development of a reliable, and appropriate animal inhalation exposure systems and the availability of human relevant animal models; (2) examination of molecular mechanisms to identify potential therapeutic targets; and (3) assessment of the long-term effects of inhaled CWAs. It is hoped that future efforts in this area will lead to the successful determination of therapeutic windows, treatment modalities, and effective medical countermeasures. A single therapeutic medical intervention likely will not be sufficient, but combinational therapies may be needed to counter the respiratory and systemic toxicities of CWAs.

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The US army experimental studies cited herein were approved by the Animal Care and Use Committee at the U.S. Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act of 1966 (P.L. 89–544), as amended. The views expressed in this article are those of the author(s) and do not reflect the official policy of the army, the Department of Defense, or the US government.

The research described in this article has been reviewed by the Center for Public Health and Environmental Assessment, EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and the policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Chapter 35

The cardiovascular system as a target of chemical warfare agents

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35.1 Introduction

The use of chemical warfare agents (Schwenk, 2018; Ciottone, 2019; Pitschmann, 2014) produces a multipronged attack on the affected organs. Alleviation requires consideration on a level that previously was not considered necessary. The presence of xenobiotics is primarily expressed by dysregulation of heart function. An early marker, changes in the electrocardiogram (ECG), is noticeable immediately. Ancillary indicators, that is, changes in the metabolites of body fluids, emerge but are delayed. Gene reprogramming, alterations in biochemical pathways, peptide hormones, and cytokines may also be present.

This section lists the usual conventional markers of a xenobiotic attack on the cardiac system. In the concluding section of this chapter, an approach to dealing with the latest and more dangerous effects of potential attack methods is presented. It points to, and describes, the therapeutic modus operandi that needs to be adopted. The propounded approach promises a heretofore unavailable insight into the dynamics of the poisoning taking place.

35.1.1 Potential indicators

To deal with the affected cardiovascular system, consideration needs to be given to one or more of the critical items listed in Table 35.1.

35.1.1.1 Troponin level changes (cTnT/cTnI)

Troponin is one of the proteins that regulate cardiac contractions. Following injury, typically when oxygen demand is not met, myocardial necrosis occurs. Egress of troponin is one of the accepted validators for myocardial infarction (MI). Located on the myofibrillar filament of the cardiac muscle, troponin contains three subunits, troponin T, troponin I, and troponin C. Excitation−contraction of the heart is regulated by the cTn complex. On contraction, the myosin filament pulls the actin filament using ATP (adenosine triphosphate) to power the contraction. The actin filament is regulated by cTnC, a calcium-binding protein, while cTnI inhibits contraction in the absence of Ca^{2+} binding to cTnC. ATPase activity of actomyosin is inhibited by cTnI. A troponin I level above 0.40 ng/mL is considered to be an indicator of probable cardiac ischemia. After damage, the myocyte sheds cTn into the circulating blood after several hours.

Troponin release is also caused by a number of other pathological conditions, in addition to coronary diseases. Decreased ventricular ejection fraction correlates with cTn elevation, as well as strenuous physical activity. In cases of renal failure, cTnT concentrations rise. Troponin increases in heart failure (HF) as well as under inflammatory conditions such as myocarditis (Korff et al., 2006; Sebastian et al., 2018).

35.1.1.2 Creatine kinase and lactate dehydrogenases, markers of tissue damage

Tissue damage is marked by the release of intracellular enzymes into the serum. Creatine kinase (CK) enzyme, found in the heart and also skeletal muscle tissue, increases due to muscle damage. Lactate dehydrogenase (LDH) enzymes, responsible for catalyzing the conversion of pyruvate to lactate, are significant markers. High levels of LDH indicate lactate accumulation and tissue damage. LDH deficiency, on the other hand, points to rhabdomyolysis, characterized by muscle necrosis with the release of intracellular constituents into the circulation. An increase in free ionized cytoplasmic and mitochondrial calcium is observed due to depletion of ATP, the usual energy

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TABLE 35.1 Selected cardiac response effects due to xenobiotics.

Activated	Effect	Measurable	References
Troponin release	Myocarditis	Myocarditis	Korff et al. (2006)
	Heart failure	Heart failure	
	Renal failure	Renal failure	
Natriuretic peptide	Bioregulation	Congestive HF	Pandit et al. (2011)
	Contractility		
	BNP upregulation		
C-reactive protein	Thrombus formation	LV dysfunction	Sproston and Ashworth (2018), McFadyen et al. (2018)
	Suppresses the thrombo-regulatory pathway	Low-grade inflammation	
Ischemia-modified albumin	Low binding for transition metals	Hypoxia, acidosis,	Oran and Oran (2017), Gaze (2009)
		Na^+-K^+ pump	
Creatine kinase and lactate dehydrogenase	Myocardial tissue damage, rhabdomyolysis	Lactate accumulation	Moghadam-Kia et al. (2016)
Parathyroid hormone	Bioregulator, myocardial contractility	Hypocalcemia, cardiac hypertrophy	Fujii (2018)

source, and goes hand in hand with dysfunction of the Na^+/K^+-ATPase and Ca^{2+}ATPase pumps. The ATP depletion causes myocyte injury and release of various muscle enzymes. An increase of intracellular calcium produces an increase in muscle cell contractility and production of reactive oxygen species (ROS).

Earlier the CK test was used to diagnose heart attacks, but has been replaced. Increased CK levels usually mark myopathies (muscular disorders) of various causes. In rhabdomyolysis, CK levels are considerably increased. Chemical and biological injury to muscles can cause rapid breakdown of muscles. Toxins act similarly.

35.1.1.3 Brain natriuretic peptide

BNPs are hormones secreted by myocytes from the cardiac ventricles in response to stretching, that is, to changes in pressure inside the heart, on cardiac stretch receptors. Upon release, BNPs activate the atrial natriuretic factor receptors NPRAs and to a lesser degree NPRBs. These decrease the vascular resistance, thereby decreasing the blood pressure. An increase in BNP designates left ventricular dysfunction that is also helpful for diagnosis of HF (Maisel et al., 2002).

35.1.1.4 C-reactive protein

C-reactive protein (CRP) is a component of a set of proteins that the immune system produces as a response to inflammatory challenges. CRP is markedly increased by trauma or infection, thereby it is a predictor of future cardiovascular events. Generally, CRP values higher than 3 mg/L suggest elevated vascular risk.

35.1.1.5 Parathyroid hormone

Xenobiotic effects may cause unexpected cardiac changes by the parathyroid hormone (PTH; Fujii, 2018; Wu et al., 2018). The PTH, produced by parathyroid cells, among other functions regulates calcium, phosphate, and vitamin D metabolism. PTH enhances myocardial contractibility through increased entry of calcium ions by activation of voltage-dependent calcium channel influx that also affects the SA node of the heart and thus the heart rate.

PTH also promotes apoptosis of cardiomyocytes. Excess PTH hormone leads to cardiac hypertrophy and HF. The PTHrP/PTH-1 receptor participates in bioregulation of cardiomyocyte function also as part of the pathological process in the myocardium. Ischemic injury activates this signaling system that induces muscle-relaxant effects (Monego et al., 2009) that influence cardiovascular homeostasis. When PTH secretion is low, hypoparathyroidism causes hypocalcemia, with immediate signatures in the cardiac function.

35.1.1.6 Ischemia-modified albumin

Myocardial ischemia (MI) produces hypoxia, acidosis, and destruction of the Na^+-K^+ pump, resulting in the formation of ischemia-modified albumin (IMA) by

cleavage of the amino acids of the HSA (human serum albumin) N-terminal. IMA has low binding affinity for transition metals (Oran and Oran, 2017). In a short period of time following MI, there is a drastic increase in the IMA level measurable before myocardial necrosis. IMA levels are now used as a biomarker (Kader and Wahab, 2017; Gaze, 2009).

Depending on the situation, the cardiac system can also be affected by various chemicals including cyanide, as discussed in Section 35.5, and arsenic and ricin discussed in Section 35.6.

The palette of military warfare agents, their modes of action at the cellular level, including a description of their expression in the changed morphology of the tissue and modified electrical state (Romano et al., 2008; Ellison, 2008), follows. The expressed effects of poisons on the heart are dose related. The references give a good summary of the expected effects of each of the anticipated conditions. ECGs (Wexler et al., 1947; Karki et al., 2004; Delk et al., 2007), illustrating the effect of the toxicants, are given where available. New biomarkers, miRNAs, short noncoding RNA sequences that regulate gene expression and control cellular processes, are revolutionizing the detection and treatment of cardiotoxicity and are discussed in a separate section. Approaches to therapy and recommended antidotes for some of the discussed toxicants are also given.

35.1.2 Hazard models

Current approaches gaging the effect of warfare agents on cardiac function assume a primary effect of the attacking medium. With new agents, several effects acting concurrently need to be considered to gage the overall impact. Initially, the Cox Impartial Hazards Model may be appropriate, but with the availability of additional data, to gage how the factors impact multivariable Cox regression analysis would be a possible approach.

This chapter describes how the heart is affected by and reacts to various chemical warfare agents (Balazs, 1981; Baskin, 1991; Acosta, 2001). The next section gives an overview of the anatomy and functioning of the heart (Opie, 1998) with an emphasis on the components most vulnerable to the presence of toxins. The importance of cardiac neuronal changes as possible indicators of exogenous effects is underscored. The normal electrical state and signature of cardiac toxicity are contrasted.

35.2 Background

35.2.1 Cardiac anatomy

The timed delivery of oxygenated blood to all parts of the body is the function of the four-chambered pump, the heart. It is enclosed in a double-layered sac, the pericardium, with the inner layer, the visceral pericardium, anchoring the heart, and the outer layer attached to the sternum. A cardiac skeleton anchors the four heart valves, the atria, and the ventricles. The thick wall separating the two ventricles, the interventricular septum, houses the Purkinje fibers, which play an important part in the electrical activity of the heart.

Deoxygenated blood from the body enters the heart by the vena cava and empties into the right atrium. Through the tricuspid valve, blood then transits into the right ventricle and via the pulmonic valve enters the lungs, where it is oxygenated. It returns to the heart through the pulmonary veins and the atrium to the left ventricle. This muscular pump redistributes the oxygenated blood to all parts of the body.

The heart is mediated by an electrical system that is easily disturbed by toxicants, which change the timing, flow, and magnitude of the electrical pulses and thus interfere with the required pumping action. The activity of the heart is myogenic, that is, the activity is initiated by the heart itself. Various components of the heart, the sinoatrial (SA) node, atrioventricular (AV) node, and the Purkinje fibers are capable of pacemaker activity. Healthy atrial and ventricular tissue does not engage in pacemaker activity. The primary pacemaker is the SA node, but in diseased or affected states the secondary pacemakers take over.

The SA node lies in the right atrium, in an epicardial location and functions as the primary source of the electrical impulse formation. It consists of specialized muscle cells, smaller than ventricular cells containing few contractile elements. Due to its location, the action potential generated traverses the atria first, causing the primary contraction. The action potential is relayed to the AV node, the conduit for the electrification of the ventricles. The signal must pass through the AV node, otherwise the atria and the ventricles are electrically insulated from each other. A time delay in transmission is needed for the atria to be emptied of blood.

The AV node is a subendocardial right atrial construct that is located within a fibrous stroma in the triangle of Koch. It is connected to both sympathetic and parasympathetic nerves. The bundle of His arises from the distal section of the AV node and goes to the summit of the ventricular septum. It is the only nonpathologic electrical conduction between the ventricles. The bundle of His divides into the left and right bundle branches, which then become the Purkinje fibers that interweave the contractile cells of the ventricle and speed the excitation throughout the ventricles.

The action potential of the cardiac myocytes is initiated by an inward sodium current in both the atria and the ventricles with inward calcium currents contributing to

the upstroke in the SA and AV nodes. Slow inward currents result in lower resting and activation potentials. In the latter, membrane potentials range from −40 to −70 mV and the activation threshold lies in the range of −30 to −40 mV and shows phase 4 depolarization. These cells are modulated by acetylcholine (ACh) and catecholamine. The duration of the action potential depends on the outgoing potassium current durations, with the inward calcium currents of lesser importance. It is longest in the Purkinje fibers. Resting potentials of atrial and ventricular cells range from −80 to −90 mV, with activation thresholds in the −60 to −70 mV range. Impulse conduction ranges up to 300 V/s in atrial and ventricular cells and up to 900 V/s in the Purkinje tissue.

35.2.2 Innervation of the heart

The autonomic nervous system guides the electrical and mechanical functions of the heart (Klabunde, 2012). The heart is innervated by both the sympathetic and parasympathetic systems, which have opposite effects and are activated reciprocally. They play important roles in arrhythmia susceptibility. Sympathetic stimulation originates from the intermediolateral column of the thoracic spinal cord. Its neurotransmitter, norepinephrine, is released from neurons of the postganglionic fibers of stellate ganglia and epinephrine is released from the adrenal medulla. Both act on cardiac β-adrenergic receptors. Beta-adrenergic receptors of the myocardium play an important role in regulation of heart function. They belong to G-protein-coupled receptors that normally bind to norepinephrine released by sympathetic adrenergic nerves. Receptors that bind catecholamine will stimulate the sympathetic nervous system, for example, increasing the heart rate.

Sympathetic nerves are predominantly on the epicardial surface. Receptors for norepinephrine on cardiac muscle are of the β-adrenergic type. Postganglionic sympathetic neurons innervate the SA and AV nodes, the conduction system, and the myocardial fibers in the ventricles. Epinephrine is the primary endogenous catecholamine produced in the adrenal medulla and regulates organic metabolism. It stimulates β_1 receptors, enhances ventricular contractility, and enhances SA nodal cell phase 4 depolarization, that is, impulse generation.

The cholinergic, that is, parasympathetic system, acts through the vagal nerves by release of ACh that opposes the sympathetic stimulation. Parasympathetic preganglionic neurons originate in the medulla. Parasympathetic fibers terminate mainly on cells of the atria. Parasympathetic innervation is denser in the SA and AV nodes than the left ventricle. The right vagus nerve innervates the SA node. The neurotransmitter ACh and adenosine promote susceptibility to atrial fibrillation and shorten atrial refractoriness. Excessive stimulation causes bradyarrhythmia. The left vagus nerve innervates the AV node, where excessive stimulation results in AV block. Receptors for ACh are of the M2 muscarinic type and ACh binding to muscarinic receptors inhibits cAMP production. ACh has a negative chronotropic effect (slows the heart), slows conduction (negative dromotropic effect), and also has a negative inotropic effect (decreases the strength of contraction). The latter is through activation of the current $I_{K,ACh}$ resulting also in a shortening of the action potential. ACh has three actions on cardiac muscle: (1) it activates ACh-sensitive K-current, $I_{K,ACh}$; (2) it inhibits the voltage, time-dependent inward calcium current I_{Ca}; and (3) it inhibits the hyperpolarization-activated inward current I_f, important for pace making. Ventricular muscle is not affected by vagal stimulation.

There are anatomical physiological differences between the sympathetic and parasympathetic systems. The parasympathetic ganglia lie within or close to the organ that postganglionic nerves innervate, while sympathetic ganglia lie closer to the spinal cord. There is limited parasympathetic innervation of the ventricles compared to the atria. The vagal nerve fibers are mostly intramural. The heightened adrenergic activation in the ventricles is potentially arrhythmogenic. All catecholamine receptors are metabotropic. They act by initiating metabolic processes affecting cellular functions. β-Adrenergic receptors, receptors for epinephrine and norepinephrine, act by stimulatory G proteins to increase cAMP in the postsynaptic cell. cAMP binds to and activates protein-kinase enzyme.

Disturbed balance between the parasympathetic and the sympathetic systems can result in disturbances in cardiac function. As discussed later, chemical warfare agents play an important role in disturbing this balance.

35.2.3 Neuropeptides

Vasoactive intestinal peptide (VIP), a neurotransmitter, is found in extrinsic and intrinsic nerves of the heart. VIP is released by the vagal nerve, and its effect is to increase the pacemaker current I_f and pacemaker rates (Chang et al., 1994; Accili et al., 1996). VIP release takes place under high-frequency stimulation. As an internal brake, it limits the ability of ACh to excessively suppress the sinus node and other pacemakers. It also has an effect on the calcium-activated potassium channel.

VIP acts as a parasympathetic neurotransmitter in its involvement in the postsynaptic control of the heart. VIP is exactly opposite in its action to that of ACh. Vagal activity, causing release of ACh, slows the heart but corelease of VIP leads to tachycardia due to VIP preferring receptors leading to an increase in adenylate cyclase and an accumulation of cAMP.

Neuropeptide Y (NPY) suppresses the pacemaker current I_f. Colocalized with norepinephrine in sympathetic nerve terminals on the heart, it is released with catecholamines during sympathetic neural activation. NPYs on cardiomyocytes have surface membrane-binding sites and suppress contractility in concentrations from 10^{-9} and above. In ventricular myocytes, the L-type Ca^{2+} current, as well as the delayed rectifier potassium currents, are suppressed.

35.2.4 Energetics of the heart

The heart requires a continuous supply of energy to be able to sustain its pumping action. Most of the energy is derived from fatty acids. Under ischemic or anaerobic conditions, glycolysis comes into play, consuming large amounts of glucose with the adverse effect of the formation of lactic acid (Jafri et al., 2001).

More than 95% of the metabolic energy is used in the form of ATP. Its concentration in a myocyte is about 10 mM. ATP is synthesized by oxidative phosphorylation in the mitochondria. There, acetyl CoA is broken down to CO_2 and hydrogen atoms. Electrons are pumped out to form a proton gradient across the mitochondrial membrane. The protons reenter the mitochondria and combine with oxygen, eventually forming water.

The glycolytic pathway under hypoxic conditions produces only a limited amount of ATP. Pyruvate formed in glycolysis is transported into the mitochondria where CO_2 is formed through cellular respiration. From each glucose molecule, 28 ATP molecules are formed in anaerobic glucose metabolism. The heart also has an energy reserve in the form of phosphocreatine (PCr), which is an immediate precursor of ATP. In the reverse Lohman reaction, CK favors the maintenance of ATP concentration at required levels. The energy released during hydrolysis of the phosphoanhydride bond in ATP powers energetically unfavorable processes, such as the transport of molecules against a concentration gradient. During conditions of hypoxia, ATP is first degraded to adenosine diphosphate, then to adenosine monophosphate (AMP), and then to adenosine. The latter diffuses into the circulating blood where the adenosine concentration rapidly increases under conditions of cellular damage. The action of adenosine is antiinflammatory as well as inhibitory.

35.2.5 Electrophysiology

The SA node, consisting of spindle-shaped cells, initiates the electrical activity of the heart. From its location in the right atrium in proximity to the superior vena cava, the electrical activity spreads to the atria whose cells are larger than those of the SA. The pulse from the atria spreads to the AV node, the gateway to the ventricles. The atria and the ventricles are electrically isolated. The AV node also slows down the electrical activity, giving the atria time to fill. The bundle of His is the upper end of the electrical path, which through the Purkinje fibers allows the electrical signal to activate the ventricles and thus to pump the blood.

Each cell's activity goes through four phases of the action potential (Grant, 2009). The morphology of each type of cell is different. Also, the kinds of electrolytes moved, and their quantity and speed, are dictated by environmental conditions, detailed in Carmeliet and Vereecke (2002). Xenobiotic interference with the transmission of the ions changes the electrical homeostasis, and commences the breakdown in the state of the tissue. The membrane currents, pump, and exchanger of importance in this regard are given in Table 35.2. Anionic chloride currents, especially those activated by swelling, play an important role in cyanide intoxication (Baumgarten and Clemio, 2003).

35.3 Signatures of cardiac toxicity

35.3.1 The electrocardiogram as a diagnostic tool for poisoning

35.3.1.1 Recorded morphological changes on the electrocardiogram

At present, morphological changes in an ECG are insufficient to make a definitive diagnosis of a poisoning but are useful in giving guidance as to which of the ion currents are affected and thus to the possible nature of the toxicity (Yates and Manini, 2012). Without access to and comparison with a preincident ECG and the multitude of factors that need to be considered, the diagnosis may be erroneous. On the other hand, poisons cause definite changes in timing and morphology of the ECG of affected individuals (Delk et al., 2007; Gussak et al., 2004; Dalvi et al., 1986; Zoltani and Baskin, 2007) and thus an ECG constitutes an important tool in the initial evaluation.

Disequilibrium in the electrolyte balance can provide diagnostic clues. For example, hyperkalemia causes tall T-waves in leads II, III, V_2 to V_4, when the potassium balance exceeds 5.5 mmol/L. In conjunction, the amplitude of the P wave is reduced and QRS is widened. Hyperkalemia is usually present when the amplitude of the T-wave is higher than that of the R-wave. With increasing potassium concentration, P-waves widen and eventually disappear. Accentuated hyperkalemia results in asystole.

TABLE 35.2 Membrane currents, pump and exchanger of importance.

Current	Function/origin	Effect
I_{Na}	Voltage-gated Na^+ current	Depolarization
I_{K}^+	Voltage-gated K^+ channel	
I_{Ca}^{2+}	L-type Ca^{2+} current	
I_{Cl^-swell}	Activated by cell swelling	Cell swelling
$I_{K,ATP}$	Activated by fall in intracellular ATP	Fall in ATP
I_f	Hyperpolarization-activated current carried by Na^+ and K^+ in sinoatrial and AV node cells and His-Purkinje cells and contributing to phase 4 depolarization	
$I_{K,ACh}$	Parasympathetic control of the heart	ACh
Na^+/K^+-ATPase	Moves Na^+ out, K^+ into cell against concentration gradient using ATP for energy	
Na/Ca	Exchanges intracellular Ca^{2+} for extracellular Na^+	Responsible for DAD

Hypokalemia results in decreased T-wave amplitude and ST-segment depression; however, accurate QT interval measurement is difficult. Malignant ventricular arrhythmias result when potassium concentrations become very low. Hypercalcemia shortens the QT interval, while hypocalcemia produces ST-segment prolongation.

Left bundle branch block is characteristic of poisoning and is defined (Zimetbaum et al., 2004) as QRS >0.12 s with delayed intrinsicoid deflection in the V_1, V_5, and V_6 leads greater than 0.05 s. The risk of arrhythmia is greatest when QRS is ≥ 0.11 s. Right bundle branch block greater than 0.12 s is a fairly good predictor of arrhythmic death.

Blockade of potassium, sodium, calcium channels, β-adrenergic receptor sites, and the Na^+/K^+-ATPase pump change the ECG and may give an indication of the type of poisoning present in the tissue (Delk et al., 2007).

Reductions in the outward potassium ion flow prolong QT, a harbinger of torsade de pointes (TdP), and ventricular fibrillation (VF). Sodium channel blockers delay the entry of sodium ions, widening the QRS complex. In extreme cases, asystole ensues. A subsidiary event may be ventricular tachycardia degenerating into VF.

For control of tachyarrhythmia, calcium channel blockers are used. Blockade of these channels decreases contractility and SA and AV node depolarization. Blocking β_1 receptor sites within the myocardium reduces the intracellular calcium concentration and contractility. The potassium concentration is increased, an undesirable effect when the β_2 receptors are blocked. Inhibition of the Na^+/K^+-ATPase pump results in $[K^+]_o$ and intercellular $[Na^+]_i$ increases, in turn increasing the intracellular calcium concentrations.

35.3.1.2 Long QT

An important predictor of arrhythmia is changes in the duration of the QT trace, the time for ventricular repolarization, displacement of the ST-segment, and changes in the pattern of T-waves that may sometimes be seen as a T and U wave (Roden, 2008). This can be linked to ventricular tachycardia, including TdP, an abnormal heart rhythm. On the ECG it is expressed by twisting of the QRS complex around the isoelectric line. Lengthened QT increases the time available for intracellular calcium accumulation, enabling early afterdepolarization (EAD) in the Purkinje fibers, and activates calmodulin (CaM) and calmodulin kinase (CaMK). CaMK is believed to enhance afterdepolarization, triggering the TdP. CaMK increases L-type calcium channel activity. Anderson (2006) reported that inhibition of CaMKII can prevent cellular arrhythmia where QT prolongation is present (Zoltani and Baskin, 2007).

During the plateau phase of the action potential, there is a delicate balance between the L-type Ca^{2+} channels and the slowly and rapidly activating cardiac delayed rectifier currents, I_{Ks} and I_{Kr} (Chen et al., 2016). Due to the slow onset of I_{Ks} activation during the rectification of I_{Kr}, a limited amount of repolarizing current flows. The L-type Ca^{2+} channels are time-dependently inactivated while an outflowing potassium current takes place. With a net outflow of ions, repolarization takes place. It is critical that the I_{Kr} deactivates slowly. Thus, small changes in the inward or outward conductance, that is, the amount of current leaving or entering, has a deciding influence on the length of the repolarization. The effect of poisons on these processes plays an important role in determining the QT-segment length.

35.3.2 Biochemical markers of tissue injury

35.3.2.1 Conventional biomarkers

Protein markers released into the bloodstream have been used as indicators of cardiac injury. The current gold-standard marker is troponin. Three types of troponin, as well as elevated CK−MB levels, and peripherally myoglobin, are used as indicators of cardiac tissue damage (Apak et al., 2005; O'Brien, 2008). Biomarkers of HF (Mittmann et al., 1998), including natriuretic peptides that increase in response to wall stress and ST2, reflective of ventricular remodeling and cardiac fibrosis, may be evident as a consequence of toxicity, and are detailed by Maisel (2012).

The cardiac troponin complex consists of three parts. Troponin T (cTnT) facilitates contraction, troponin I (cTnI) inhibits actin−myosin interactions, and troponin C binds to calcium ions. Troponin I and T are specific to the heart but cTnT is also expressed by injured skeletal muscle. In the course of cell damage, cardiac troponin is released from myocytes, facilitated by increased membrane permeability that allows smaller troponin fragments to traverse the membrane. Complicating the use of troponin levels is the fact that in cases where there is cardiac injury without cardiac cell membrane disruption, serum troponin levels can increase. Also, altered ion homeostasis may not be reflected in troponin levels.

In cancer patients undergoing high-dose chemotherapy, elevated troponin I and CK−MB are predictors of ventricular systolic dysfunction, and thus indicators of cardiac damage. Immunological ultraviolet-array is used for determination of the level of CK−MB, where an elevation greater than twice normal is taken as an indicator of myocardial infarction. Cardiac troponin is measured by electrochemiluminescent immunoassay. Cardiac impairment is indicated by the presence of troponin. The release of the enzyme has been linked to a mismatch between oxygen demand and expenditure in the tissue, lessening of the time available for diastolic coronary perfusion, catecholamine release, and intense sympathetic stimulation. Warfare agent-associated cardiac toxicity is characterized by these conditions.

Is the increased level of troponin due to damage to the heart muscle? In-depth studies note that stroke victims without acute myocardial infarction have elevated troponin levels. Severe renal dysfunction may also cause it. Thus, until convincing proof is presented for noncardiac causes, an increase in the presence of marker enzymes can be taken as an indicator of damage due to toxins in the cardiac tissue.

CK is a protein necessary for ATP generation. One of its forms, CK−MB, is found mainly in the myocardium and upon tissue damage, such as myocardial infarction (MI), becomes elevated. It takes up to 24 h for the elevated level to reach its peak. A difficulty resides in the fact that unlike troponin, an assay does not distinguish between cardiac and skeletal muscle damage. Also, in about a third of MI cases, while CK−MB levels stay neutral, troponin elevation is noted. The normal level of troponin in the blood for troponin I is less than 10 μg/L and troponin T less than 0.1 ng/mL, while for CK−MB it is less than 3.0 ng/mL. For humans, normal CK is in the range of 55−170 IU/L (international units per liter) but is less specific than CK−MB for cardiac tissue damage.

35.3.2.2 miRNA

Traditional biomarkers lack tissue specificity—and their appearance in body fluids is time-delayed. For example, troponin levels are elevated between 3 and 12 h after myocardial injury. Recently microRNAs have gained acceptance as alternate biomarkers (Sahu, 2013; Gidlof et al., 2013; Vickers et al., 2014; Yamakuchi, 2012). Their tissue specificity, sensitivity, stability, and timeliness underlie their usefulness and have opened new therapeutic approaches. These noncoding RNAs play an important role as gene expression regulators, and miRNAs regulate fundamental cellular functions and toxicological outcomes. The number of identified miRNAs is large and regulates the majority of mRNAs.

miRNAs are short, 22 nucleotides long, and modulate protein expression by binding to mRNA, thereby inhibiting translation or targeting them for degradation. As regulators of physiological processes, they play an important part in the developing pathology (Kim, 2013; Vacchi-Suzzi et al., 2013).

Biogenesis of miRNAs starts in the nucleus of the cell where miRNA genes are transcribed by RNA polymerase into primary miRNAs. The Drosha enzyme then creates the precursor miRNA. After export to the cytoplasm, cleavage by the Dicer enzyme produces the miRNA. The mature miRNA is incorporated into RISC, the RNA-induced silencing complex. The miRNA of the RISC complex binds to the target messenger RNAs, preventing translation. After myocardial infarction, for example, miRNA levels increase signaling-reduced systolic function, where the left ventricle ejection fraction is $<50\%$. Microvesicles shed from the cell, that is, exomes, export the miRNAs and transport them to other locations, in effect constituting the intercellular communication and thereby regulating genetic function and protein generation (Romaine et al., 2015).

In drug toxicity (Yokoi and Nakajima, 2013), as well as oncology, miRNAs are becoming useful tools in making diagnoses. As shown in Section 35.7, miRNAs offer new therapeutic approaches for warfare agent-caused cardiac toxicity. For further details on cardiac toxicity biomarkers, readers are referred to Zoltani (2019).

35.4 Indices of the toxicity of warfare agents

35.4.1 Classes of warfare agents

Three broad classes of warfare agents are considered: organophosphate nerve agents (Newmark, 2007; Munro et al., 1994), the cyanides (Baskin et al., 2009), and a third general category that is less weaponized but whose effects are important as potential terror agents, the arsenics and ricin. An excellent overview of chemical warfare agents is given by Marrs et al. (2007).

Respiratory tract irritants, such as phosgene and vesicants, and including sulfur mustard, have a subsidiary cardiac impact but the mode of disability is acute lung injury.

The classes of nerve agents of primary interest are the "G" agents, first synthesized in Germany. Denser than air, these agents hug the ground and represent a vapor hazard due to their volatility. Tabun (GA), sarin (GB), and primarily soman (GD) are those most widely used. "V" (venomous) agents were developed in the United Kingdom. VX is more toxic than the G agents. Cyanides are less weaponized but also of concern. Arsenic and ricin are more terror threats than battlefield weapons. Novichok, developed in the Soviet Union, is the most potent nerve agent but there is little public information available.

35.4.2 Background

Chemically induced cardiac failure has been the subject of a number of works. Baskin (1991) and Acosta (2001) give an overview of the subject. Hypoxia is one of the effects of the decreased availability of ATP, which depresses muscle contraction. The energy that is supplied by the phosphate bonds is possible only as long as aerobic glycolysis and oxidative phosphorylation are maintained. Under anoxic conditions, this is no longer possible and with the adrenergic stimulus continuing, calcium accumulation in the mitochondria leads to impairment of function, eventually causing dose-related lesions (Suzuki, 1968).

35.4.3 Signatures of toxicity

Warfare agent-supplied xenobiotics disturb cardiac homeostasis. Changes of concern include the following:

- Morphological tissue changes;
- Enhanced neural stimulation;
- Release of neuropeptides;
- Activation of usually dormant cardiac currents;
- Alteration in the storing and movement of ions;
- Disturbances in energy use and storage.

Ferrans et al. (1969) noted ultrastructural changes in myofibrils, including mitochondrial swelling and disorganization. Catecholamines also produce changes in the activity of oxidative enzymes. The decline in the activity progresses to the point where necrosis is evident. Ancillary effects include loss of myocardial potassium and an increase in interstitial fluid. Intracellular calcium overload also develops, as noted by Fleckenstein et al. (1974). It has been hypothesized that necrosis due to catecholamine overload may be caused by a defect in energy supply needed for the maintenance of cellular processes.

At low concentrations, the catecholamines, epinephrine, and norepinephrine exert positive inotropic effects on the myocardium. High concentrations, however, can cause cardiac lesions (Inoue et al., 1998). Even physiologic concentrations, when extended over time, lead to cardiac damage, as shown by Szakacs and Mellman (1960). The LD_{50} of norepinephrine in rats is 680 mg/kg, but at doses as low as 0.02 mg/kg, focal necrotic lesions are produced.

The oxidation product of catecholamine is adrenochrome, whose accumulation has also been linked to myocardial necrosis, as well as morphological and subcellular alteration (Yates and Dhalla, 1975). Administration of adrenochrome to rats also induced heart arrhythmias. Free radicals may also contribute to these processes. It has been suggested that catecholamines may activate β-adrenergic receptors, stimulating adenylate cyclase, and thus elevating cAMP. This in turn activates protein kinase, increasing the phosphorylation of slow calcium channels, possibly resulting in an overflow of calcium, leading to necrosis preceded by swollen sarcoplasmic reticulum, altered enzymatic activities, and lower ATP.

Cyanide primarily blocks oxidative phosphorylation and ATP production. Every heart beat uses up to 2% of the energy available to the cell. Arsenic primarily causes a long QT (LQT) interval on the ECG, by blocking the fast potassium current, an action that is a precursor to VF. The activation of dormant currents by the presence of xenobiotics, including cell swelling, radically changes the electrical homeostasis of the tissue (Zoltani and Baskin, 2007).

35.4.4 Nerve agents

There is a difference in distribution of nerve agents for different organs of the body as well as different locations within the heart. Roth et al. (1993) detail the effects on the heart. Aspects of neurotoxicity are discussed in detail in Omahen (2011).

35.4.4.1 Mechanism of action

Nerve agents are OP compounds, which irreversibly inhibit AChE, leading to ACh accumulation, and cause overstimulation of muscarinic and nicotinic ACh receptors.

TABLE 35.3 Toxicities of weaponized agents.

Agent	LCt_{50} (mg min/m^3)	ICt_{50} (mg min/m^3)	LD_{50} (mg/kg)
GA	400	300	14.28
GB	100	75	24.28
GD	70		5.0
VX	50	35	0.1428
Cyanide	2500–5000		1.1

The effect at the SA node, the primary heart control site, is inhibitory and bradycardia results. VX primarily affects neurotransmitter receptors, those of norepinephrine, and also affects the central nervous system unrelated to AChE inhibition. The balance between nicotinic receptor potentiating effects and muscarinic effects at parasympathetic postganglionic fibers determines the effect of nerve gases on the heart.

Toxicants change the homeostatic distribution and timing of the presence of electrolytes, enzymes, and other constituents of myocytes and their environment. These changes affect and determine the current flow, in turn affecting the electrical state and possibly signaling the onset of electrical instability. Table 35.3 shows toxicities of weaponized nerve agents.

35.4.5 Electrocardiographic signature of organophosphates

Disturbances in the electrical activity of the heart caused by xenobiotics are readily discernible in a surface electrocardiogram (Yurumez et al., 2009; Dalvi et al., 1986; Chuang et al., 1996). OPs cause QT prolongation on the ECG that subsequently can degenerate into TdP. In one reported OP case, 79.7% had QT prolongations with ST-segment and T-wave abnormalities (Karki et al., 2004; Rubinshtein et al., 2002). Changes are ultimately expressed in arrhythmia, VF and TdP, and severe disturbance of the energy homeostasis of the heart. Second-degree AV heart block as well as ST-T wave alterations (Balali-Mood and Saber, 2012) have also been observed.

An initial indicator of an impending arrhythmia is prolongation of the QT interval. The lengthening can be caused by a reduction in the outward currents or increased inward currents, that is, imbalance of inward and outward currents during the second and third phases of the cardiac cycle, namely, the I_{Kr}, I_{Ks}, $I_{Ca(L)}$, and I_{Na} currents. These currents can generate EAD and trigger activity at the end of repolarization. Primary sites are the Purkinje fibers and the mid-myocardial M cells. In the Purkinje fibers, at higher positive resting potentials than the ventricles, blockade is voltage dependent, with an increased block in the depolarized tissue. The failure of complete repolarization leads to dispersion of refractoriness and enhanced arrhythmogenicity. Acquired LQT interval involves pause-dependent or short–long–short RR interval sequences on the ECG, and enhancement of sympathetic nervous system tone. LQT is favored in cases of severe bradycardia, hypokalemia, and conditions that lead to EAD. LQT may not be a sufficient condition for TdP. Stimulation of adrenergic receptors plays a significant role but it can enhance or inhibit afterdepolarization.

The electrical activity of the heart is modulated by hormones and neurotransmitters. Xenobiotics disturb their balance. The parasympathetic system releases ACh and the sympathetic system releases catecholamines (norepinephrine and epinephrine). These bind to α and β types of receptors. α_1-Receptors are present on the postsynaptic member of the organ and mediate vasoconstriction and stimulation of Na^+/K^+-ATPase, the Na^+/Ca^{2+} exchanger, and the Na^+/H^+ exchanger. This affects the I_{KATP} and inhibits the I_{Na^+} and I_{to}. The α-receptor stimulation thus effectuates depolarization and the α_2-receptor inhibits norepinephrine release.

Cardiac β_1-receptors and atrial and ventricular β_2-receptors take part in a positive inotropic response. Inhibition of catecholamines at β-adrenergic receptor sites interrupts the production of cAMP and inhibits calcium influx, producing a negative inotropic effect that yields a reduction in heart rate.

The main effect of adrenergic stimulation is to enhance the intracellular adenylyl cyclase activity. This in turn increases cyclic AMP levels. Protein kinase A is modulated and activates calcium and potassium channels. Phosphorylation of the calcium channel increases the inward current leading to EAD.

β_1-Adrenergic stimulation increases the activity of the Na^+/K^+ pump and inhibits EAD. Hyperpolarization of resting membrane potential counteracts automaticity, leading to a decrease in heart rate. α_1-Adrenergic stimulation hyperpolarizes membranes, and enables EAD and TdP by blocking potassium channels. Release of norepinephrine enhances α_1-adrenergic stimulation, facilitating EAD (Schomig et al., 1995). In long QT, increased sympathetic discharge may induce both bradycardia and TdP. Increased adenosine increases ventricular refractoriness and sympathetic tone, setting the conditions for reentrant arrhythmias. Hypokalemia prolongs cardiac action potential and may be the precursor of EAD. The net outward potassium currents are reduced and the inward calcium current is increased.

35.4.5.1 Toxic effects of organophosphates on the heart

The anticholinesterase effects of OP nerve agents depend on the sites where they act. The muscarinic effects in the heart act through the parasympathetic system, while the nicotinic effects act through the sympathetic system. Agents like tabun, sarin, and soman, analogous to acetylcholine, are capable of changing the receptor sites, which increases the conductance of electrophysiological signals related to the enhancement of neuromuscular function. VX, on the other hand, counteracts the effect of ACh, interrupting neuromuscular function.

ACh is a neurotransmitter that is present in the parasympathetic nervous system and is stored in vesicles. Its function, under normal circumstances, is terminated by AChE that is present in both the pre- and postsynaptic membranes. OPs inhibit AChE by electrophilic (a reactant which accepts an electron pair from a molecule with which it forms a covalent bond) attack of the enzyme. The neurotoxicity of OPs was exploited in the development of nerve gases. The key to the understanding of OP inhibition of AChE is the enzyme's serine hydroxyl group behavior. Normally, it attacks ACh at the carboxyl carbon yielding a covalent bond between the enzyme and the ACh substrate. The transiently acetylated enzyme is next hydrolyzed and the active enzyme site is regenerated. The active serine hydroxyl group is attacked by the electrophilic phosphorus of the OP instead of the ACh. A covalent, nonhydrolyzable bond is formed between the enzyme and the OP, leaving the enzyme in an inactivated form. Release of an alkyl chain further strengthens the phosphorus−enzyme bond. With AChE inactivated, the amount of ACh present increases, resulting in overstimulation of the tissue. The pathophysiological effects that result can be explained by the overabundance of ACh. Overstimulation of muscarinic receptors in the heart leads to bradycardia.

35.5 Specific warfare agents of concern regarding the heart

35.5.1 Currently the most widely used agents rely on organophosphate compounds

The heart may be affected by both muscarinic and nicotinic effects. In the former, stimulation of the parasympathetic nerve endings occurs, while in the latter, excess ACh on the nicotinic receptors is of importance. The cardiovascular effects are tachycardia caused by overstimulation of the sympathetic system, bradyarrhythmia, AV block, hypotension and QT prolongation, VF, and TdP (Grmec et al., 2004).

OP intoxication manifests itself in three phases. First, a nicotinic phase hypertension and sinus tachycardia occurs. This is followed by sinus bradycardia and parasympathetic overstimulation and ST-segment changes on the ECG and rhythm disturbances. During the last phase, TdP and sudden cardiac death occur. According to Ludomirsky et al. (1982), a QTc (QT corrected for heart rate) of 580 ms signals high probability of sudden cardiac death. Roth et al. (1993) give further insight from actual cases.

A breakdown of actual cardiac symptoms for OP poisoning in hospital admissions is given by Karki et al. (2004). Sixty-seven percent of the acute OP cases had QT prolongation, 24% experienced ST-segment elevation, and 17% had inverted T-waves. Nine percent had atrial tachycardia, 9% ventricular tachycardia, and 4% had VF. Sinus tachycardia was observed in 35% of admissions, while sinus bradycardia was noted in 28%. Noting that acidosis and electrolyte derangement play a major role in the development of cardiac events, they recommend "atropine in adequate doses very early in the course of the illness" as the strategy to be implemented. Table 35.4 summarizes the effect of OP on the electrophysiology of cardiac tissue.

OPs are known to induce time-delayed neurotoxicity. This is due to the inhibition of an esterase in nerve tissue, neuropathy target esterase (NTE), that is also found in muscle and blood cells. The NTE level in the blood is an indicator of the inhibition of the enzyme. Inhibition of NTE and aging, the process of following the OP binding to an active esterase site that prevents the reactivation of the site, is important for selection of an antidote against certain OP nerve agents. It is of primary concern for novichok. There is little information available on its neurotoxicity and cardiac toxicity.

35.5.1.1 VX

The most toxic nerve agent available in the Western world is VX. It is an inhibitor of AChE, which acts by increasing the ACh at the nerve synapses. Toxicity sets in when more than 50% of the AChE enzyme is inhibited. The $AChE_{50}$ value for VX is taken as 0.023 mg/kg for an

TABLE 35.4 Effect of OP on the electrophysiology of cardiac tissue.

AChE	Lesions				Second messenger (VIP, others)
	Anoxia	Acidosis	Modulated ion concentration	Release of catecholamines	
ACh overload causes bradycardia, slows conduction in AV, prevents hydrolysis $[Ca^{2+}] \uparrow \rightarrow I_{K,ACh} \uparrow$	Lowers ATP, cAMP; ATPase inhibition $I_{K,ATP} \uparrow$ (activated), AP shortened	pH ↓, Na^+/H^+ exchange ↑, [ATP] ↓, $[K^+]_o \uparrow$, reduces I_{Kr} by increasing rate of deactivation, shifts voltage dependence of activation to more positive potentials, $g_K \uparrow$	$[K^+]_o \uparrow$effect on velocity of propagation, inexcitability	Prolongs AP, $[Na^+] \uparrow$, Na^+/K^+-ATPase antagonized DAD enhanced difference for α, β receptors	Adenylate cyclase activation
Antagonizes adenylyl cyclase		Cytoplasmic $[Ca^{2+}] \uparrow$slows repolarization, reduces max. diastolic potential	$[Na^+] \uparrow Na^+/K^+$ pump inhibition, Na^+/Ca^{2+} exchanger $\rightarrow Ca^{2+}$ influx	α stimulation: reperfusion arrhythmia, calcium overload, gap junction conductance ↓, exchanger stimulation, activates Na^+/K^+ pump	$I_f \uparrow$, cAMP ↑, → HR ↑OP reduces cAMP $\rightarrow Ca^{2+}$ influx, inhibits adenylate cyclase, stimulates ATP, $I_K \uparrow$, affects $I_{Ca(L)}$, EAD, DAD → arrhythmia
Arrests cAMP synthesis, depresses I_f (pacemaker current)		$I_{Ca(L)} \downarrow$, $I_{Na} \downarrow$ (inactivation of fast Na^+ channel), decreased excitability, CO_2 accumulation	$[Ca^{2+}] \uparrow Na^+/Ca^{2+}$ exchanger ↓reduced SR uptake		
ACh inhibits adenylate cyclase			$[Mg^{2+}] \uparrow$(hydrolysis of ATP), activates enzymes, reduces $I_{Ca(L)}$, I_{K1}, $I_{K,ACh}$, I_{KATP}, I_{Ks}	β stimulation: adenylate cyclase, elevates cAMP, increases calcium influx, I_f activation, triggered activity improves modal conduction	

[...], Concentration; ↑, increase; ↓, decrease; →, yields; $I_{(.)}$, ionic current with the subscript denoting the channel type; *DAD*, delayed afterdepolarization; *EAD*, early afterdepolarization; *g*, conductivity of the tissue; SR, sarcoplasmic reticulum.

oral dose (Sidell, 1974). Activity of RBC-AChE (red blood cell acetylcholinesterase) and plasma butyrylcholinesterase (BChE) are other markers of toxicity of OPs. In tests on human volunteers, 1 μg of VX/kg by i.v. infusion showed a depression in AChE activity of more than 50%, but for these tests no cardiac symptoms were recorded. VX produces intense stimulation of nicotinic ACh receptor ion channels and muscarinic ACh receptors. Though not recorded in human volunteer tests, cardiac effects are known to take place, based on animal studies. VX exposure produces positive inotropic effects. Arrhythmia was noted in rats and dogs (Robineau and Guittin, 1987). In guinea pigs treated with VX, delayed afterdepolarization was found (Corbier and Robineau, 1989). In rats the effect of VX is ascribed to inhibition of the cardiac Na^+/K^+-ATPase α_1 isoform. At 1 μM concentration, the inhibition is 35% (Robineau et al., 1991). Physostigmine, hyoscine, and HI 6 were investigated as antidotes (Wetherell et al., 2007; Munro et al., 1994).

35.5.1.2 Tabun

GA, a unitary chemical munition, inhibits AChE, the enzyme responsible for the breakdown of the neurotransmitter ACh. When inhaled, its toxicity is half that of sarin. It depresses plasma and RBC-AChE activities significantly in the blood. At 20%−25% of red blood cell AChE baseline, the effect of the nerve agent becomes noticeable. There is no evidence of systemic toxicity other than the cholinesterase activity (Parker et al., 1990; Munro et al., 1994).

GA has not been shown to produce OPIDN (organophosphorus-induced delayed neurotoxicity) except at extremely high doses. The cardiac effect of GA conforms to OP-caused arrhythmias and AV block.

35.5.1.3 Sarin

Sarin was involved in terrorist attacks in Japan. The increase in sympathetic and parasympathetic tone results in tachycardia, ST-segment modulation (Abraham et al., 2001), and arrhythmia. Recently, Watanabe et al. (2013) also described acute effects of a sarin-like OP agent, bis (isopropyl methyl) phosphonate, on cardiovascular parameters in artificially ventilated, anesthetized rats. Findings revealed that powerful stimulation of sympathetic and parasympathetic nerves resulted in modulation of heart rate and blood pressure. Inhibition of cholinesterase within the neuroeffector junction also affects nerve impulse transmission by direct action. Direct action on muscarinic or nicotinic ACh receptors (Somani et al., 1992) is observed when the blood level of sarin exceeds the micromolar level. Sarin inhibits RBC-AChE 80%−100% as well as plasma-BChE between 30% and 50% (Grob and Harvey, 1958). It also binds to another serine-containing enzyme aliesterase or carboxylesterase, thereby reducing its concentration for AChE inhibition in rats (Gupta et al., 1991).

Sarin exhibits OP-delayed cardiotoxicity possibly due to epinephrine-induced arrhythmias (Allon et al., 2005; Morris et al., 2006). This hypothesis is supported by Khositseth et al. (2005) who showed that epinephrine changes T-waves in the ECG where AT prolongation already exists.

35.5.1.4 Soman

The most widely used nerve agent, GD, exerts a defining effect on cardiovascular function. Myocardial degeneration and necrosis were noted by Britt et al. (2000) in soman-exposed rhesus macaques. Generally, upon contact, bradycardia and modulated cardiac output are followed by hypotension and changes in the ECG. Notably, AV conduction modulation, QT extension, T-wave and ST-segment changes characteristic of myocardial infarction, and ACh-induced coronary vasospasm are noted. For OP's cardiac effects, important references include Sidell (1974), Kiss and Fazekas (1983), and Anastassiades and Ioannides (1984). McKenzie et al. (1996) also showed that in swine a dose of $2 \times LD_{50}$ soman i.v. increased coronary sinus plasma ACh by 314% and decreased coronary blood flow to 55% of control. The evidence indicates OP-caused deaths are due to ACh-induced coronary vasospasms that culminate in MI. This is seen as VF on the ECGs. The Food and Drug Administration (FDA) approved pyridostigmine bromide as a pretreatment for soman poisoning (Newmark, 2007).

35.5.1.5 Novichok

In open literature, little is known about these agents developed in the Soviet Union. They are assessed to be 5 to 10 times more toxic than VX (Ellison, 2008; Chai et al., 2018). The toxicity of these binary agents does not rely primarily on the inhibition of AChE, but it is thought that it causes permanent neuropathy. Consequently, conventional nerve agent antidotes may not ameliorate its toxicity. Reactive oximes such as potassium 2,3-butanedione monoximate may be useful in detoxification. No published information is available on cardiac pathologies caused by novichok agents, but general effects of its use in terrorist poisoning have been published (BBC, 2018).

35.5.2 Antidotes for organophosphate nerve agents

Enzymatic hydrolysis (where enzymes cleave bonds in molecules with the addition of water) is a primary route for elimination of nerve agents. Specifically, treatment for OP intoxication includes atropine, a muscarinic receptor antagonist, an anticonvulsant such as diazepam, and a cholinesterase reactivator, an oxime. It has been found that drug-induced inhibition of ACh release and accumulation in the synaptic cleft, such as adenosine receptor antagonist early in OP intoxication, improves the chances of survival. Some AChE reactivators, such as bispyridinum oximes, HI 6 and HLö 7 with atropine, are quite effective. Oximes can reactivate acetylcholinesterase by attaching to phosphorus, subsequently divorcing from the AChE enzyme. A large number of other oximes were also investigated (Balali-Mood and Saber, 2012).

35.5.3 Cyanide

Classified as a blood agent, cyanide is usually deployed as hydrogen cyanide and cyanogen chloride. Considerable literature exists on the effects of cyanide (Suzuki, 1968; Baskin et al., 2009). Cyanide binds irreversibly to its target sites. In the human host it preferentially accumulates in the hypothalamus and neural tissue. Its concentration in red blood cells is much greater than in plasma. The lethal dose is of the order of 1 mg/kg or inhalation of 50 mL of hydrogen cyanide gas.

35.5.3.1 Toxicity

Cyanide binds to Fe^{3+} in heme-containing proteins. This inhibits the terminal cytochrome complex IV of the electron transport chain. The block lock of complex IV by cyanide depletes ATP, culminating in cell death. Oxygen is unable to reoxidize the reduced cytochrome a3. Thus, cellular respiration is inhibited as well as ATP production, in essence depriving the cells, tissue, and ultimately the whole body of oxygen. Hypoxia evolves into metabolic

acidosis and decreased oxygen saturation. The extent of lactic acidosis indicates the severity of the cyanide poisoning. In a collapsed individual, plasma lactate is an indicator of cyanide poisoning. In severe cyanide poisonings, up to 98% of the cyanide in the bloodstream is tightly bound to red blood cells. The ancillary response is myocardial depression and decline in cardiac output. Bradycardia, hypotension, and cardiac arrhythmia then develop into VF and cardiovascular collapse.

In severe cyanide poisonings, autonomic shock due to the release of biogenic amines plays a role. The coronary arterial vasoconstriction, resulting in an increase in central venous pressure, leads to the observed shock-like state that is not attributable to inhibition of cytochrome oxidase. In the liver, CN is metabolized by rhodanese to thiocyanate, which is excreted in the urine.

Hypoxia is one of the signatures of cyanide poisoning. In CN poisoning, as in ischemia, oxidative metabolism is blocked and acidosis is enhanced. Acidosis depresses contractility and metabolism, while sparing ATP supplies.

A dose of 0.54 mg of hydrogen cyanide per kg of body weight is fatal, with an average of 1.4 mg. Data indicate that the heart absorbs the second highest amount of cyanide per organ weight. On the other hand, in sublethal exposure, cyanide-fed rabbits (Okolie and Osagie, 2000) do not show the hemorrhaging in the cardiac tissue noted by Suzuki (1968). Cyanide also causes a decline in $[K^+]_i$, that is, significant hypokalemia and an increase in $[Na^+]_i$. These changes were not reflected in the skeletal muscle. Cyanide caused a decline in ATP, the energy source of the cell, to less than 10% of the normal value, which activates the otherwise dormant potassium channel and the outward current I_{KATP}. These changes result in shortening of the AP and a decrease in the contraction.

Cyanide also causes endogenous catecholamine release (Schomig et al., 1995; Inoue et al., 1998). Inoue et al. (1998) also point out that cyanide-produced depolarization increases intracellular calcium due to the suppression of the potassium channels and activation of the voltage-dependent calcium channel. Anoxia induces suppression of the sodium pump and activates cation channels due to the decrease in ATP. A further consequence of the presence of cyanide in the tissue is inhibition of the Na^+/Ca^{2+} exchanger (NCX) (Ju and Allen, 2005). NCX is important for the pacemaker currents. Metabolic inhibition of NCX reduces the firing rate of pacemaker cells.

Cyanide poisoning is marked by metabolic acidosis and a large anion gap. The latter is a consequence of the blocked oxidative phosphorylation and the increased rate of glycolysis. Maduh et al. (1990) showed that cyanide also affects H^+ and thus the pH of the tissues. In turn, the Ca^{2+} transport process is disrupted, leading to a rise in cystolic $[Ca^{2+}]$. Acidification depolarizes the cell membrane and changes the potassium conductance.

Cyanide also exerts a strong influence on the vagus nerve and thus on the VIP that under normal conditions exerts a strong inotropic and chronotropic effect. VIP stimulates adenylyl cyclase activity. In ventricular myocytes, VIP potentiates voltage-gated Ca^{2+} channel currents and also acts on pacemaker currents.

As shown by Goldhaber et al. (1991), contractile failure, that is, twitch shortening, is caused by cyanide. It is said to be due to failure of activation of the Ca^{2+} current. Electrocardiographic manifestations of CN poisoning are shown in Katzman and Penney (1993), and Wexler et al. (1947) described in Zoltani et al. (2004), and summarized in Table 35.5.

The ECG of an individual (Wexler et al., 1947) executed by inhalation of cyanic acid revealed that, initially, between the first and third minutes, a heart rate slowing was discernible with the disappearance of the P-wave. Later the heart rate increased slightly. T-waves showed an increase in amplitude and a marked shortening of the ST-segment. One subject, unlike some others in this cohort, showed normal AV conduction until ventricular tachycardia and VF developed.

35.5.3.2 Antidotes for cyanide poisoning

Bhattacharya and Flora (2015) and Cummings (2004) described the following approaches:

1. Use of a nitrite (oxidizing agent such as sodium nitrite) to change the ferrous ion of hemoglobin to a ferric ion. The created methemoglobin binds cyanide forming cyanmethemoglobin. One drawback, however, is that impairment of oxygen transport occurs, i.e. that is, the amount of hemoglobin available is reduced. Amyl nitrite to generate methemoglobin is no longer preferred, since it does not bind enough cyanide.
2. Sulfur donors for conversion of cyanide to thiocyanate by rhodanese or other sulfur transferases, that is, a source of sulfur. For moderate poisoning, sodium thiosulfate is the usual choice.
3. Use of cobalt chemistry that chelates the cyanide directly, such as hydroxocobalamin.
4. Hydroxocobalamins, precursors to vitamin B_{12}, are preferred in France and elsewhere in Europe. Hydroxocobalamin binds cyanide to form cyanocobalamin. It does not interfere with tissue oxygenation but large doses are required to be effective.
5. In the United Kingdom, dicobalt edentate, which chelates the cyanide directly, is preferred, but assurance that cyanide poisoning is present is needed since this antidote contains cobalt, which can be toxic.

TABLE 35.5 Effect of CN on the electrophysiology of cardiac tissue.

P	QRS	ST	T	Remarks	Reference
Disappearance (auricular arrest)	Change in amplitude, right axis deviation	Shortening, disappear	Incr. amplitude, origin of T-waves on QRS	0.11–0.20 mg/kg NaCN, inhalation, AV dissociation, HR↓, BBB, initial bradycardia, AV block, asystole	Wexler et al. (1947) (man)
	Lengthened	Absent or depressed, elevated after 20 s	Increased, decreased over time, T starts on top of R, diphasic, negative	0.4–0.8 mg/kg NaCNHR↓, bradycardia, Wenckebach, heart block, V-flutter, VF	Leimdorfer (1950) (cats)
		Short, shifted toward T-wave	Tall T, surpassing R, sometimes inverted	0.1 mg/kg NaCN, bradycardia, incomplete AV blockade, VF	Paulet (1955) (dog)
	Abnormal	Elevated, shortened	T-wave beginning high on QRS	0.3–0.6 g sodium nitrite as antidote with sodium thiosulfate, atrial fibrillation	De Busk and Seidl (1969) (man)
	Narrow QRS	Nonspecific ST-T changes in anterior chest leads, shortened ST-segment	Fusion of T-wave into QRS, sharp rise of T-wave	Sinus tachycardia, acidosis with high anion gap	Chin and Calderon (2000) (man)
	Q-wave present in lead III, persisted after treatment of acidosis	Deviation of 4 mm in leads II, III, aVF, 2 mm in V_6, ST depression of 2–4 mm in V_1–V_4, I, aVL leads, normalization of ST-segment in precordial leads	T-wave inversion in II, III, aVF, V_5 and V_6 leads	Acidosis, CN level of 3 μg/mL, anion gap present	Sanchez et al. (2001) (man)

↑, Increase; ↓, decrease.

35.6 Other terror agents

35.6.1 Arsenic

Arsine blood agents were first developed for battlefield use during World War I. Due to difficulties with dispersion, they were never used. Arsenic, however, has potential use as a terror agent (Ellison, 2008; Sidell et al., 1997).

Arsine is the simplest compound of arsenic. It is colorless and 2.5 times denser than air with an odor resembling garlic. Arsine binds to hemoglobin of the red blood cells, destroying them. Poisoning kills by allosteric inhibition of metabolic enzymes. Arsenic disrupts ATP production. Arsenic inhibits pyruvate dehydrogenase, uncoupling oxidation phosphorylation. Arsenic poisoning also occurs through arsenic–oxygen compounds, especially arsenic trioxide, As_2O_3 (ATO), which is 500 times more toxic than pure arsenic (Yamazaki et al., 2006).

ATO has been effectively used as a remedy for relapsed acute promyelocytic leukemia, but with the side effect that it causes QT interval prolongation, possibly heralding ventricular arrhythmia (Chiang et al., 2002). Abnormalities in $I_{Ca(L)}$ in myocytes were also noted (Sun et al., 2006). ATO's direct effect on cardiac repolarization with its effect on I_{Kr} was noted by Haverkamp et al. (2000). ATO also causes cellular Ca^{2+} overload and augments APD (action potential duration) (Yamazaki et al., 2006). Chronic arsenic exposure leads to QT prolongation, blockage of I_{Kr}, TdP, and T-U alternans, and changes in the T-wave result (Little et al., 1990; Ficker et al., 2004).

It has been suggested that potassium ion channel alteration induced by arsenic may be related to hERG (Vandenberg et al., 2012), trafficking defects. ECG changes in arsenic poisonings have been reported by Fennel (1981). The T-waves are domed. ECG changes,

especially where arsenic involvement is not severe, are reversible. Hemodialysis and BAL (dimercaprol) therapy (to remove excess lead) have been found to be effective. Sun et al. (2006) suggest that choline can normalize QT interval abnormality by inhibiting $[Ca^{2+}]_i$ and $I_{Ca(L)}$ in ventricular myocytes when ATO is present. Arsenic intoxication results in widened QRS by 0.06 s and prolonged QT (Ahmad et al., 2006). Ventricular tachycardia and VF have been reported by St Petery et al. (1970).

35.6.2 Ricin

Ricin (*Ricinus communis*), a toxic glycoprotein derived from the castor bean, causes hypotension and myocardial hemorrhage. The Centers for Disease Control and Prevention (CDC) lists it as a category B agent due to its easy availability as a terrorist weapon. Only a limited amount of information is available in the open literature on ricin's effect on the heart. The medical files of Georgi Markov, the Bulgarian journalist, assassinated in London with what was assumed to be ricin, are not publicly available (Crompton, 1980; Aggarwal et al., 2017). He suffered complete atrioventricular conduction block.

Ricin is a glycoprotein made up of two chains linked by a disulfide bond. Its toxicity results from one of the chains inhibiting protein synthesis by irreversibly inactivating eukaryotic ribosomes. The lethal dose of ricin has been set at 1−20 mg/kg of body weight (Bradberry et al., 2003). Christiansen et al. (1994) performed extensive experiments on rabbits. Their main findings include the following:

1. Ricin caused vasodilatation and increased endothelial-dependent vascular relaxation resulting in hypotension.
2. Ricin disturbed calcium homeostasis leading to cell necrosis.
3. Ricin reduced both systolic and diastolic left ventricular function.
4. Ricin caused myocardial hemorrhage.

Balint (1974) and later Zhang et al. (1994) found that at the lethal dose in rabbits, ricin caused hemorrhage and necrosis. Christiansen et al. (1994) found that the release of norepinephrine from sympathetic nerves in the vasculature is not impaired by ricin. The CDC, under signs and symptoms of ricin poisoning that may be encountered, cite cardiovascular collapse (hypovolemic shock).

ECG abnormalities in children who ingested castor beans have been noted by Kaszas and Papp (1960). These include QT interval lengthening, repolarization changes, and intraventricular conduction disturbances. Crompton (1980) later reported on experiments on pigs that experienced hemorrhagic lesions and an abnormal ECG due to ricin. Genes of ricin toxicity have been identified suggesting therapeutic targets (Moreau et al., 2011). At the present time, no antidotes or effective therapy are available to counteract the effects of ricin.

35.7 Therapeutics under development

Chemical warfare agents almost instantaneously affect the cardiac system. miRNA signatures give rapid and conclusive evidence of the affected site and degree of involvement. Predominantly, though not exclusively, the injury turns off genes, expressed through miRNAs. Thus, modulating the genetic response to overcome dysregulation of miRNAs, therapy aims to normalize miRNA expression, silence those that are overexpressed, and replace those that are downregulated.

The heart expresses a large number of miRNAs (Wang, 2011; Zhou et al., 2018) that have been correlated with the genes that affect the heart's varied functions, including electrophysiology. Many miRNAs have a large number of target genes. The change in the value of the particular miRNA from normal reflects not only the injured organ but also the degree of injury. Thus, for example, changes in the ECG can be related to particular miRNAs.

A common sign of cardiac injury is up- or downregulation of miRNAs. An effective countermeasure is inhibition of the affected miRNAs. AMOs (anti-miRNA oligonucleotides), single-stranded 2′-*O* methyl-modified oligonucleotides fragments that are antisense to its target miRNA have been used. The methyl group improves the binding to RNA. AMO acts by base-pairing, producing loss-of-function of the miRNAs. Multitarget AMO (MT-AMO) enables a single AMO fragment with the capability of targeting multiple miRNAs. miRNA sponges are similar, but contain multiple binding sites for an miRNA seed family.

miRNA inhibitors bind to complementary mRNA sequences that result in posttranscriptional gene silencing. Engineered oligonucleotides, antagomirs, and miRA erasers and sponges are currently preferred. Anti-miRNAs contain the reverse sequence of a mature miRNA that is able to reduce the endogenous levels of the miRNA. In addition, it must be cell permeable, stable, and able to bind to the selected miRNA with high probability.

miRNAs play a role in cancer pathogenesis. miRNAs that function as tumor suppressors may be downregulated by disease while oncogenes are upregulated. mRNA targets of these miRNAs have been identified. This suggests that genes involved with atrial and VF can also be modulated. In fact, miR-1 changes in the myocardium of normal hearts have induced arrhythmias (Kim, 2013; Su et al., 2017).

TABLE 35.6 Warfare agent-induced cardiac pathologies.

Cardiac syndrome	Physical syndrome	Dysregulated miRNA	Cellular expression	References
Heart failure	Inability of heart to pump sufficient blood	miR-423-5p	Siderophages generated	Sahu (2013), Mittmann et al. (1998)
		miR-18b-3p		
		miR-129-5p		
		miR-1254		
		miR-675		
		mir-622		
Ischemia	Restricted blood flow to tissues	miR-1	ATP level lowered, surge in reactive oxygen, necrosis, pH decline	Gidlof et al. (2013), Vickers et al. (2014)
		miR-133a		
		miR-208a		
		miR-499		
Modulated ion flow	ECG changes, arrhythmia	miR-1	Membrane excitability, conduction changes, modulated repolarization	Grant (2009)
		miR-133		
Vascular	Inflammation, vasodilation, hemorrhage	miR-126	Endothelial cells promote vascular homeostasis, release factors including NO	Yamakuchi (2012)
		miR-17		
		miR-146a		

In view of a quantifiable association of miRNA and gene expression, cardiac injury caused by warfare agents may be amenable to therapies based on miRNAs.

HF, ischemia, modulated ion flow, and vascular inflammation have specific associated miRNAs, as shown in Table 35.6. A pathological condition, caused by an injury, may reflect several conditions, thus the miRNA expression profile may not be unique.

Ischemia upregulates several miRNAs, including the miR-15 family. As shown by Hullinger et al. (2012), oligonucleotides can effectively suppress expression of miR-15, reduce infarct size, and remodel after ischemic injury.

Tissue-specific regulation of miRNAs has been achieved by adenoviruses, enabling continuous replacement. It has also been successfully used in gene therapy to overcome tumor genesis. Considerable detail on miRNA therapy is given by van Rooij (2011) and Thum (2011).

In replacement therapy, to restore the loss of function caused by a warfare agent, miRNA mimics can be used to reintroduce miRNAs into affected cells that also reactivate pathways and target silenced genes. Lipoproteins would be the preferred means to introduce the encapsulated miRNA to achieve these ends.

For resolution of warfare agent-caused cardiotoxicity, miRNA approaches, with specificity and timeliness, offer a myriad of opportunities.

35.8 Concluding remarks and future directions

Per organ weight, the heart is the second most preferred depository for several of the warfare agents used. Disturbance of the electrical homeostasis, the cellular energy production, and breakdown of the electrical control network within the tissue are mostly affected.

35.8.1 Current concerns

Areas that need further insight include the following:

- Chemical warfare agents cause miRNA concentration changes in body fluids; tissue specificity and time of appearance of the changes after exposure need to be correlated with conventional biomarkers and electrophysiologic markers, such as ECG changes;
- Means of reactivation, that is, overcoming "aging" caused by OP-based agents;
- Nanoparticles that carry medicines show promising results; their use to counteract xenobiotic poisoning needs to be exploited;
- Role of NTE in cardiac toxicity.

Human data for various cardiotoxic scenarios is unavailable. The means of prompt identification of the particular xenobiotic causing poisoning remains an urgent

task. Progress in metabolomics, the identification biomarkers that result from metabolic changes caused by the presence of xenobiotics, will enable the development of chip-based rapid-responding assaying devices. Hopefully, this chapter will be an incentive to follow up on the approach outlined here that has tremendous potential for the understanding and treatment of cardiac toxicity.

35.8.2 Potential future scenarios

Advent of weapons systems that create changes beyond muscarinic and nicotine effects pose new challenges to the preservation of cardiac function. Synthetic biology-enabled weapons produce biochemicals by novel metabolic pathways and modification of the human microbiome. Use of CRISPR, the gene-editing tool, by means of viruses can cut human DNA and cause, among others, cardiac misfunction. Dysbiosis and disease as a weapon need to be considered as outlined in detail in National Academies of Sciences, Engineering, and Medicine (2018) and by Dando (2015).

Morphological changes leading to an alteration in cellular ionic balance affect automaticity and conductivity as well as unequal parasympathetic and sympathetic activity leading to possible arrhythmia.

Peptide bioregulators that control normal biological processes, like toxins, are weaponizable substances. Tachykinins cause rapid loss of blood pressure. Endothelin is a potent vasoconstrictor peptide that stimulates the contraction of blood vessels. Toxins are quick-acting blockers of cellular metabolism affecting conduction of nerve impulses. T-2 mycotoxin or the botulinum toxins are more dangerous than soman or VX.

35.9 A new approach

The effectiveness of the newest chemical warfare agents has undergone remarkable improvements. The altered miRNA induced by these compositions evokes or silences genes that make the ensuing tissue activity more difficult to counteract. To determine an effective therapy, the miRNA signature produced by the attacking substance must be known. miRNAs regulate gene expression at the posttranscriptional level by binding to the 3' untranslated region of the target mRNAs. miRNA is tissue specific and molded by the transcription process. Cardiac-specific miRNA expressions and signatures for specific cardiac dysfunctions (Ikeda et al., 2007; Small et al., 2010) are known.

Signatures of dysregulated miRNA of affected tissue need to be determined. The potential damage is remedied by modifying the newly established miRNA. Among the available choices, it can be determined by "next-generation sequencing." It is one of the preferred techniques (Shendure and Hanlee, 2008; Liu 2018) although it is elaborate. It needs to turn off genes that the xenobiotic turned on and substitute by replacement a new miRNA, that could also be a synthetic miRNA, an miRNA mimic. Implementation offers several possibilities. Use of nanoparticles, as in drug delivery, is an option. Use of inorganic material carriers, such as ferric oxide or positively charged thiolated gold or magnetic nonviral nanoparticles (Schade et al., 2014), has been proposed. Thereby the introduced miRNA restores the suspended and desired miRNA activities.

Essentially, the procedure consists of three steps:

1. Identify the miRNA of the system that the xenobiotic created;
2. Delete the miRNA that was introduced;
3. Determine and install the desired miRNA.

This procedure has not been previously used on cardiovascular systems targeted by chemical warfare agents.

Considerable new information, currently not used, could upgrade therapeutic processes. Recently, circular RNA has received attention. Formed by back-splicing of one or two exons, it results in a covalently closed molecule. Known as circular RNA (circRNA), they participate in cardiac pathophysiological processes and also have been proposed as biomarkers. CircRNA can also act, by interacting with RNA-binding proteins, as miRNA sponges to regulate transcription.

The xenobiotic effect of circRNA in cardiac tissue needs to be explored. These tissue- and pathologic-specific molecules are more stable than linear RNAs and protected from degradation by exonucleases. Exploration of the biogenesis and function of circRNAs, a potential biomarker in cardiovascular disease, is an active area (Aufiero et al., 2019; Gomes et al., 2018; Holdt et al., 2018).

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Chapter 36

Ocular toxicity of chemical warfare agents

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36.1 Introduction

Vision is the dominant sense in humans. In healthy individuals the visual system contributes to approximately 80% of perception and cognition. The eye and the accessory visual structures (e.g., the conjunctiva, lacrimal apparatus and eyelids) are developmentally and structurally complex, and proper vision is critically dependent on the functional integration of diverse tissues with distinct morphological and cellular properties. The intricate internal architecture and exposed location of the eye render it highly susceptible to injury by a wide array of toxins and toxicant agents, with the severity of injury influenced by factors such as dose, physicochemical properties, and environmental conditions. Manifestation of injury is further modulated by the specific interactions of each agent with the various physiological and biochemical microenvironments of the eye. In some cases, recovery from intoxication may be critically influenced by the regenerative capacity of individual ocular tissues. In this chapter, we summarize what is known about the toxicological and toxicokinetic effects of ocular exposure to a wide array of traditional chemical warfare agents (CWAs), including several biological toxins. Although the primary concern is usually the long-term protection of vision, even CWAs that manifest with short-term and reversible ocular symptoms can cause a temporary incapacitation that has profound effects on lifestyle. In some cases, acute ocular symptoms may serve as prodromic markers of exposure or predicative of future pathophysiologies. Thus, understanding the toxicological mechanisms underlying both acute and chronic ocular responses to CWA exposure is critical to predictive diagnosis of the potential impact on the visual apparatus and development of therapeutic strategies.

Traditionally, CWAs are divided into seven general categories based on their toxicological properties: vesicants, biological toxins, blood agents, tear agents, nerve agents, incapacitating agents, and choking agents (Table 36.1; Romano et al., 2008). Vesicants are a class of highly reactive alkylating compounds that catastrophically disrupt cellular functions by forming covalent adducts with proteins, ribonucleic acid, and deoxyribonucleic acid. In contrast, organophosphorus nerve agents (OPNAs) block acetylcholinesterase (AChE) activity, causing synaptic accumulation of acetylcholine (ACh) and overstimulation of cholinergic receptors. The resulting cholinergic toxidrome potentiates sympathetic signaling, resulting in distinctive but reversible changes in the pupillary apparatus, the lens, and possibly the retina. Incapacitating agents work in a variety of ways; antimuscarinics such as 3-quinuclidinyl benzilate (BZ) elicit an anticholinergic toxidrome, disrupting vision through loss of accommodation, decreased depth of field, ciliary muscle paralysis, and mydriasis. Blood agents interfere with oxygen delivery and oxygen utilization, causing acute cytotoxicity due to progressive tissue hypoxia and necrosis. The tear agents are nonlethal, lacrimating agents that rapidly irritate mucosal membranes and peripheral ocular nerves, causing an acute discomfort that is typically transient and reversible. The conjunctiva is particularly susceptible to blood agents and tear agents because of its exposed location and extensive innervation. Ocular toxicities can also result from exposure to diverse biological toxins, including botulinum neurotoxins (BoNTs), a family of potent neuroparalytic toxins produced by the *Clostridium* genus of bacteria; ricin, a transcriptional blocker extracted from the castor bean; and *Streptococcus* enterotoxin B (SEB), a bacterial superantigen. Finally, we propose a toxidromic-based approach for classifying chemical toxicities with the goal of assessing acute and chronic risks and simplifying treatment strategies.

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TABLE 36.1 Representative CWAs of each major category, including biotoxins and incapacitating agents.

Vesicants		**Blood agents**	
Mustard gas	HD	Hydrogen cyanide	AC
Nitrogen mustard	HN	Cyanogen chloride	CK
Lewisite	L	**Tear agents**	
Phosgene oxime	CX	Mace	CN
Nerve agents		CS gas	CS
Tabun	GA	CR gas	CR
Sarin	GB	OC gas	OC
Soman	GD	**Psychomimetic incapacitating agents**	
Cyclosarin	GF	3-Quinuclidinyl BZ	BZ
VX	VX	**Biological toxins**	
VR	VR	Botulinum Neurotoxins	X
Novichok		Ricin	W
Choking agents		Staphylococcal Enterotoxin B	UC
Phosgene	CG	Saxitoxin	TZ
Diphosgene	DP	Tetrodotoxin	PP
Chlorpicrin	PS		

36.2 Background

36.2.1 The structure of the eye

The eye is constructed of three layers (or tunics) which play key roles in enabling the entry and visual processing of light. Each layer is separated by fluid-filled chambers which provide nourishment as well as structural support (Fig. 36.1). The outer layer of the eye includes the sclera, conjunctiva, cornea, and corneal limbus. As a result of their exposed locations, these tissues are primary targets for CWAs. The white, opaque sclera is a tough fibrous connective tissue layer containing interlacing type I collagen bundles and elastic fibers that give the eye its shape. The anterior surface of the sclera is covered by the conjunctiva, a layer of stratified columnar epithelial cells that produce mucus to help lubricate the eye. The conjunctiva is richly supplied with blood vessels and is highly susceptible to inflammation. At the front of the eye is the cornea, a transparent and avascular yet highly innervated tissue. The cornea is responsible for protecting the eye against insults such as injury and infection. It also provides roughly two-thirds of the total refractive power of the eye and is therefore the major refracting lens of the eye (Meek et al., 2003). The corneal limbus, which is located at the junction between the conjunctiva and cornea, plays an essential role in sustaining and repairing the corneal epithelium. Limbal cytotoxicity, therefore, has

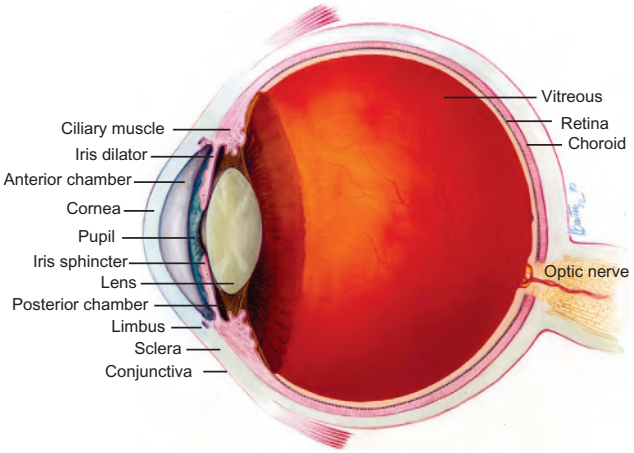


FIGURE 36.1 Anatomy of the eye.
Diagram of the human eye, labeled with the structures involved in ocular toxicity following exposure to CWAs. *Modified with permission from National Eye Institute, National Institutes of Health, reference number NEA04.*

severe consequences to corneal function, as discussed in the next section.

Between the cornea and the uveal tissues is the anterior chamber, which contains a watery fluid called the aqueous humor (AH). The anterior chamber exerts positive pressure against the posterior corneal surface (a.k.a., intraocular pressure) that shapes the cornea. Together the

cornea and the AH form an outer lens that plays the most significant role in refracting light toward the center of the eye. The AH also provides nourishment for the tissues lining the anterior chamber, and consequently is rich in metabolites (Goel et al., 2010). The AH is continuously secreted by the ciliary epithelium at a rate of 3 μL/min (in humans), and flows from the posterior chamber through the iris into the anterior chamber, exiting the eye primarily via the trabecular meshwork, where it is absorbed into the bloodstream. The entire volume is replaced every 90 min (Goel et al., 2010). Penetration of CWAs through the cornea results in intracameral exposure, which can cause systemic toxicity and/or produce biomarkers through adduct formation between CWAs and intracameral proteins that subsequently can be detected in plasma (Romano et al., 2008; McNutt et al., 2012a,b). AH production and drainage are critical to normal ocular function, and even short-term functional disruption has profound negative effects on corneal integrity and vision. Long-term disruption can result in complete loss of vision.

The middle layer of ocular tissues includes the choroid, iris, lens, pupillary apparatus, and ciliary body. General inflammation of these tissues is called *uveitis* or *iritis*. The choroid is the inner vascular layer of the eye, underlying the sclera. The primary function of the choroid is to provide oxygen and nutrients to the retina. While the high degree of choroidal vascularization renders the choroid prone to inflammation, it is largely isolated from direct exposure to most CWAs as a consequence of its deep intraocular location. The narrow space delineated by the iris, lens, and ciliary structures is known as the posterior chamber. The iris is a contractile diaphragm that controls the pupillary aperture and therefore the amount of light that enters the eye. Pupil size is regulated by two iris muscles: the pupillary sphincter, which is responsible for constriction of the pupil, and the pupillary dilator, which is responsible for dilation of the pupil. These muscles are innervated by sympathetic ("fight or flight") and parasympathetic ("rest and relax") nerves that determine constriction and dilation, respectively. The lens is a crystalline structure that focuses light on the retina. The autonomic nervous system controls focus (accommodation) by changing the shape of the lens under the control of ciliary muscles. Since changes in pupil size and accommodation are principal ocular symptoms associated with exposure to many CWAs, neuro-ophthalmic processes are described in greater detail in the section below.

The majority of the volume of the eye consists of the vitreous chamber, which is filled with a transparent, gelatinous mass of extracellular matrix material similar to the corneal stroma. This mass, which is known as the vitreous humor, holds the retina in position. The retina is comprised of 10 layers of nervous tissue containing millions of hierarchically organized light receptors that are connected to the brain by the optic nerve. Because of the physicochemical barriers that reduce CWA penetration to the rear of the eye, the retina is not susceptible to CWA toxicity, with the exception of systemically distributed neurotoxic CWAs that affect retinal function and information processing.

Finally, the ocular adnexa (not shown) are tissues that provide a supportive environment to the globe. Adnexal tissues include the lacrimal glands, which are located beneath the outer portion of the upper eyelid, the eyelids, and extraocular muscles. The lacrimal glands produce tears that help lubricate and moisten the eye and flush foreign matter that may contact the surface of the eye. They also contain proteases and cytokines that regulate the activation and fate of stromal cell populations upon disruption of the epithelial barrier. The eyelids facilitate corneal wetting, protection, and flushing, while the extraocular muscles control convergence, allowing the eyes to synchronize and to provide binocular vision.

36.2.2 Effects of ocular structure on regenerative capacities

The cornea and conjunctiva are generally the first ocular tissues encountered by external agents, and thus are the most common target of chemical toxicities. In fact, the extent of CWA absorption, penetration, and reactivity with conjunctival or corneal tissues is a major determinant of ocular injury (Jester et al., 2001; Maurer et al., 2002). Corneal function is critically dependent on the ability of the corneal epithelium and corneal endothelium to maintain stromal deturgescence by controlling fluid ingress at the anterior and posterior margins of the cornea, respectively. Disruption of either barrier results in corneal edema, increased production of proinflammatory mediators, and entry of matrix-active enzymes into the stroma. Disruption of both boundaries can have devastating effects on vision, eliciting a persistent corneal edema that may progress to corneal degeneration, and ultimately require surgical intervention (Eagle et al., 1989; Alomar et al., 2011). Agents that disrupt the full-thickness integrity of the cornea can cause diverse corneal pathologies, including stromal scarring, endothelial toxicity, recurrent corneal lesions, and corneal perforation (Fig. 36.2).

Given the susceptibility of the cornea to chemical toxicity and the devastating consequences of corneal dysfunction to proper vision, the regenerative capacity of the cornea represents the most significant repair mechanism for preservation of vision after ocular exposure to CWAs. The cornea is composed of five histologically distinct layers with different regenerative capacities: corneal epithelium, Bowman's layer, stroma, Descemet's membrane

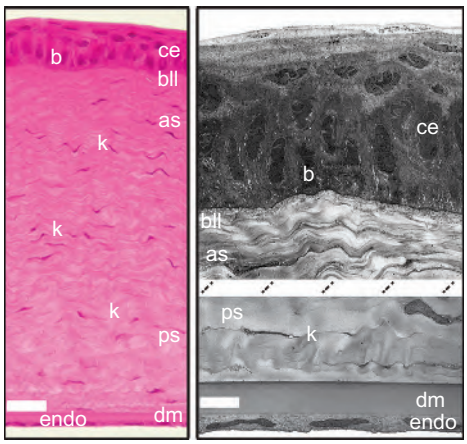


FIGURE 36.2 Structure of the cornea.
(Left) Representative histology of a rabbit corneal section. Scale bar is 40 μm. (Right) Transmission electron microscopy of a rabbit eye, demonstrating the ultrastructure of different corneal layers. Scale bar is 4 μm. ce, corneal epithelium; b, basal epithelial cell; bll, Bowman's-like layer; as, anterior stroma; k, keratocytes; ps, posterior stroma; dm, Descemet's membrane; endo, corneal endothelium. Unpublished data.

(DM), and corneal endothelium (Fig. 36.2). The corneal epithelium is a stratified epithelium with five to seven cell layers that provides a dynamic physical barrier between the external environment and intraocular space. Tight junctions among the different layers of corneal epithelial cells form a highly impermeable barrier to tear film and microorganisms (Klyce, 1972). The basal epithelial cells have several important functions, including the secretion of matrix factors, establishment of hemidesmosomal attachments to focal adhesion complexes in the anterior stroma, and proliferation to generate new epithelial cells. Anterior to the basal epithelial cells are the wing-like suprabasal cells. Over time, the suprabasal cells move superficially and terminally differentiate into the outer layers of squamous epithelial cells. These cells are shed from the ocular surface during normal wear and tear and are replaced by the steady superficial migration from underlying epithelium. Thus, homeostasis of the corneal epithelium is critically dependent on mitotic renewal.

Regeneration of the corneal epithelium primarily relies on a small population of limbal epithelial stem cells (LESCs) located in the basal region of the limbus. Although LESCs slowly cycle during homeostasis, they can become highly proliferative in response to corneal injury, undergoing asymmetric division to produce daughter transit amplifying cells that migrate into the basal layer of the corneal epithelium and undergo limited proliferation to replace corneal epithelial cells lost during normal exfoliation or due to injury (Ordonez and Di Girolamo, 2012). The limbal niche is highly vascularized, and factors that regulate niche function are delivered via multiple sources, including systemic circulation, tear film, AH, support cells in the LESC niche, keratocytes in the stroma, and corneal epithelial cells. Adverse conditions, such as chemical injury or persistent inflammation, can disrupt niche function, leading to a constellation of symptoms known as *limbal stem cell deficiency* (*LSCD*; Ahmad, 2012; Osei-Bempong et al., 2013). Dysregulation of the LESC niche has severe consequences for the corneal epithelium, which in turn evokes symptoms of corneal failure, such as invasion of goblet cells from the conjunctiva into the cornea, neovascularization, chronic inflammation, recurrent corneal erosions, keratitis, corneal ulceration, and stromal scarring.

Beneath the corneal epithelium is the corneal stroma, which encompasses over 99% of the corneal volume. The stroma is a highly ordered tissue composed of tightly packed collagen fibrils that are structured by proteoglycans into lamella (Hassell and Birk, 2010). This uniform packing is essential for rendering the stroma transparent to light. Maintenance of the stroma in a deturgescent state by the barrier functions of the corneal endothelium and corneal epithelium is a key factor in preserving lamellar organization. Disruption of either barrier increases corneal hydration and disrupts lamellar structures, causing aberrant light refraction through the stroma (Eagle et al., 1989). Under normal conditions, the stroma is sparsely populated by keratocytes, a quiescent population of mesenchymal cells that secretes transparent corneal material. Keratocytes exhibit variable responses to corneal injury, depending on their proximity to the injury (West-Mays and Dwivedi, 2006; Wilson, 2012). After a penetrating injury to the cornea, nearby keratocytes undergo apoptosis, prompted by the stromal influx of signaling molecules from the tear film or AH. More distant keratocytes undergo a fibroblastic transformation, become active, begin to proliferate, and start synthesizing matrix metalloproteinases that assist in tissue remodeling and/or scar formation (Hassell and Birk, 2010).

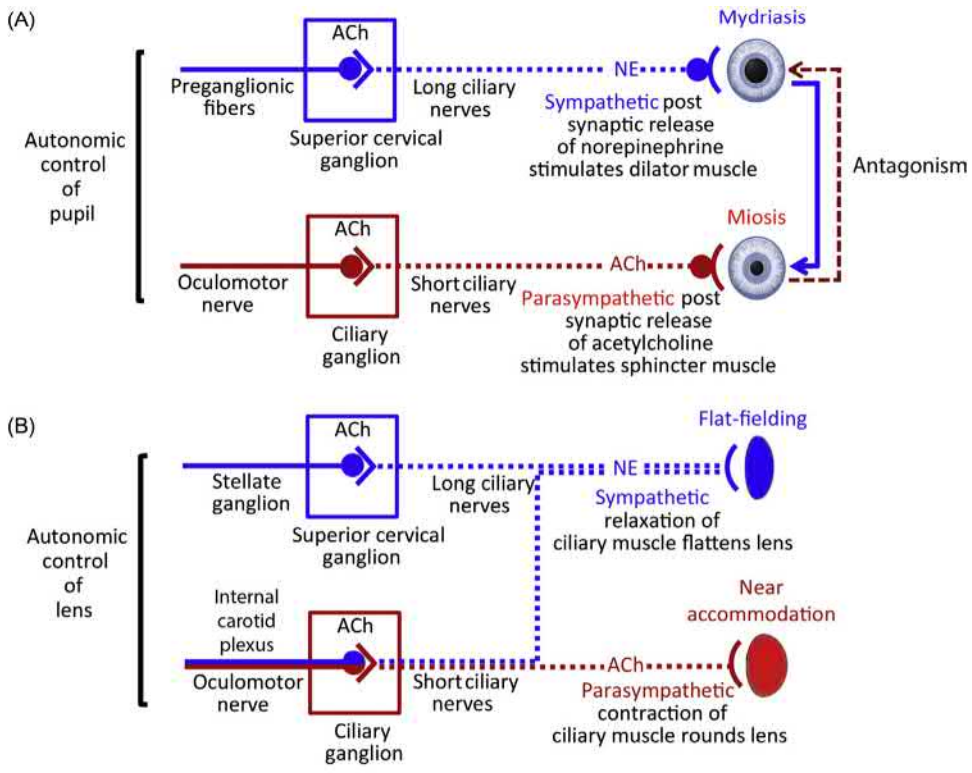
The corneal endothelium is a monolayer of cells on the DM that regulate corneal nutrition and turgescence by balancing a semipermeable barrier activity with active ion transport mechanisms. Since adult human corneal endothelial cells (CECs) do not appear to proliferate in vivo, the corneal endothelium has limited ability to recover from cytotoxic injury (Joyce et al., 1996; Senoo and Joyce, 2000). Under normal conditions, gaps in the CEC monolayer are rapidly filled by the spreading of proximal cells, driven by morphologic changes that compensate for endothelial cell loss. Once the focal CEC density falls below the threshold level required for endothelial function (estimated to be 1000−2000 cell/cm^2) (Joyce et al., 1996), the corneal endothelium can no longer maintain the stroma at the proper level of turgescence, resulting in a persistent corneal edema that, in turn, causes secondary keratopathies such as anterior segment inflammation,

epithelial bullae, and LSCD (Eagle et al., 1989; Petroll et al., 1995). Thus, while endothelial function can be restored after a mild injury by CEC spreading, recovery from more severe endothelial lesions may be limited by the repair capacity of the human endothelium.

36.2.3 Importance of neurological function to vision

Ocular function is extensively integrated with afferent and efferent neurological activity. For example, autonomic neurons control the activity of intrinsic ocular muscles (the sphincter pupillae, the dilator pupillae, and the ciliary muscle), which determine focal accommodation and pupil size. Autonomic neurons also control the function of the lacrimal glands. Neuronal afferents in the eye include sensory neurons from the conjunctiva and cornea, reflexive contributions to the iris, ciliary muscle, and eyelids, and the densely innervated retina. Finally, extraocular muscles and eyelids are controlled by cholinergic motor neuron inputs.

Under normal conditions, pupil size and focal accommodation result from a balance between sympathetic (adrenergic) and parasympathetic (cholinergic) nerve inputs (Fig. 36.3) (Levin and Kaufman, 2011). Autonomic control over pupil dilation is mediated by sympathetic postganglionic neurons originating from the superior cervical ganglion and synapsing with the iris dilator muscle. Stimulation of the sympathetic pathway results in the release of norepinephrine onto α-adrenergic receptors on the sphincter dilator, dilating the pupil and increasing the activation of light receptors in the retina. The short ciliary parasympathetic nerves project from the ciliary ganglion to form cholinergic synapses with the iris sphincter. Stimulation of the parasympathetic pathway results in pupillary constriction (miosis), reducing light input and causing tunnel vision. The dilator and sphincter muscles are also antagonistic, such that inhibiting one pathway allows the other pathway to become dominant. The system is further complicated by differential expression of stimulatory and inhibitory receptors in the uveal muscle tissues, presumably coordinating the complex



(C) Representative effects of neuro-ophthalmic agents on autonomic nervous system balance

- Organophosphorus nerve agents potential cholinergic signaling, shifting the balance toward parasympathetic dominance.
- BZ block cholinergic signaling, shifting the balance towards sympathetic dominance.
- Low doses of systemic botulinum block cholinergic release, shifting the balance toward sympathetic dominance.
- High doses of systemic botulinum block cholinergic and adrenergic release, producing tonic and poorly responsive pupils that are not dominated by either autonomic pathway.

FIGURE 36.3 Autonomic nervous system inputs to the pupil and the lens.
Diagram depicting the balance between sympathetic and parasympathetic signaling to the pupil (A) and lens (B), and summarizing how agents causing neuro-ophthalmic toxicity can alter this balance (C).

interplay of sympathetic and parasympathetic inputs (Whikehart, 2003).

Control over lens accommodation by the ciliary muscle is coordinated with pupil size and binocular convergence to optimize focus. The ciliary muscle receives both parasympathetic and sympathetic inputs from the ciliary ganglion via the short ciliary nerves. Postganglionic sympathetic signals result in the release of norepinephrine onto the ciliary muscle, activating β-adrenergic receptors and relaxing the muscle, which in turn optimizes refractive power for far vision. In contrast, parasympathetic activation activates muscarinic receptors, causing ciliary muscle contraction and rendering the lens more spherical for near-focus.

Given the extensive role that neurological function plays in proper vision, it is not surprising that exposure to CWAs that directly or indirectly alter neurological behaviors can result in ocular symptoms (Table 36.2). In fact, CWAs can be grouped into three general categories based on pathogenic mechanisms of ocular toxicity: (1) nonspecific, cytotoxic mechanisms; (2) nonspecific, noncytotoxic mechanisms; and (3) specific disruption of neuro-ophthalmic functions (Table 36.2). This novel grouping is useful in assessing the toxidromic progression (and treatments thereof) associated with each group of agents. For example, ocular exposure to Category 1 cytotoxic agents involves a common set of symptoms, including proinflammatory responses, chemosis, corneal lesions, corneal edema, limbal dysfunction, and the possibility of corneal ulceration at very high doses. Independent of the etiogenic agent, clinical management of these symptoms is essentially the same: supportive care to reduce the acute injury followed by pharmacologic and surgical treatments to facilitate corneal repair. Exposure to Category 2 agents requires supportive care, but rarely warrants more extensive regenerative therapies. In contrast, Category 3 neuro-ophthalmic modulators manifest by modulating either the activities of innervating ocular neurons or the ability of effector tissues to respond to neuronal signals. This modulation can be specific to particular neuronal subtypes, such as with cholinergic agonists or antagonists, or can be nonspecific, such as with blood agents. Although countermeasures for Category 3 toxidromic agents would be dependent on the type of agent, one common treatment might be drugs that antagonize cholinergic signaling to reverse myosis.

TABLE 36.2 Agents grouped by nature of ocular manifestations.

Category 1: Ocular irritants, cytotoxic
Vesicants
Blood agents, aerosol delivery
Choking agents
Ricin
Staphylococcal enterotoxin B
Category 2: Ocular irritants, noncytotoxic
Tear agents
Category 3: Ocular neuromodulation
Nerve agents
Tetrodotoxin
Saxitoxin
Botulinum
Blood agents, systemic
BZ

Italicized items represent entire agent categories.

36.3 Ocular toxicities of specific chemical warfare agents

36.3.1 Selection of agents discussed

The list of CWAs, CWA derivatives, and potential CWAs with ocular toxicity is lengthy, and providing a comprehensive review is beyond the scope of this chapter. To constrain this discussion, representative CWAs were chosen from (1) traditional chemical and biotoxin threat agents identified by the US Department of Defense (DOD) and the Centers for Disease Control and Prevention (CDC); (2) Schedule 1A−3A substances, as described in the 1993 United Nations Convention on the Prohibition of the Development, Production, Stockpiling, and Use of Chemical Weapons and on their Destruction, also known as the 1993 Chemical Weapons Convention (CWC); and (3) representative chemicals of toxidromic families. While only a subset of potential CWAs are discussed, the overall objective of this chapter is to consider these limited examples as part of a generalizable approach to classify and treat ocular exposure to novel chemicals with shared physicochemical properties and/or toxidromic effects.

36.4 Vesicants (Group 1)

Sulfur mustard, nitrogen mustard, and lewisite are CWC Schedule 1A substances. These substances have few legitimate applications, if any. The largest section of this chapter will focus on the vesicant class of CWAs for three reasons. First, the vesicants pose the most significant threat to the eyes, with the development of irreversible, vision-threatening injuries following severe exposures. Second, the clinical, biological, and functional

consequences of ocular exposure to vesicants have been extensively researched. Third, the vesicants have a high potency against ocular tissues, and ocular injury can occur at doses that produce minimal systemic or cutaneous effects. Given the importance of eyesight in the sensory armature, ocular exposure to vesicants can incapacitate victims for days to months and incur a profound degree of psychological distress.

36.4.1 The mustard gases

Sulfur mustard (HD; $C_4H_8Cl_2S$) and nitrogen mustard (HN-1; $C_6H_{13}Cl_2N$) are highly reactive adducting compounds that exert their cytotoxic effects through a combination of genotoxicity, production of reactive oxygen species, direct interference with protein function, and disruption of metabolism. Although the mustards are well known for their cutaneous blistering effects, the ocular injury is clinically distinct from the cutaneous injury, influenced by factors unique to the eye, including structure, biochemical composition, and regenerative mechanisms. The tactical implications of HD use on troop morale and combat capability are poignantly captured in the John Singer Sargent painting *Gassed*, based on the artist's first-hand observations of a German mustard gas attack on British troops on August 21, 1918, in which he depicted lines of vesicant-blinded troops moving slowly toward casualty stations (Fig. 36.4). Harry L. Gilchrist, the medical director of the Gas Service, US Army Expeditionary Force, described a similar phenomenon following a mustard attack on US soldiers (Gilchrist and Matz, 1933):

> *At first the troops didn't notice the gas and were not uncomfortable, but in the course of an hour or so, there was marked inflammation of their eyes. . . . by the time the gassed cases reached the casualty clearing station, the men were virtually blind and had to be led about, each man holding on to the man in front with an orderly in the lead.*

The combination of HD's physicochemical properties, latent period of injury, and severe clinical effects rendered HD to be the most effective CWA during World War I, earning it the moniker of "king of battlefield gases" (Fitzgerald, 2008; Ganesan et al., 2010). HD was responsible for 77% of all gas injuries in World War I (Hughes, 1942). Large-scale deployment of HD during World War I and the Iran–Iraq War (1980–88) resulted in well over 600,000 casualties, with estimates of 75%–90% of HD casualties presenting with ocular injuries (Papirmeister et al., 1991). HD was also deployed in a number of smaller conflicts between 1917 and 1997, including by Egypt against North Yemen during 1963–67, Italy against Ethiopia from 1935 to 1940, and the Soviet Union against China in 1934 and again in 1936–37, although there is significantly less information about the consequences of HD use during these conflicts (Tuorinsky, 2008).

36.4.1.1 Toxicokinetics of the acute ocular mustard injury in human victims

Eyes are roughly 10-fold more sensitive to HD injury than are skin or pulmonary tissue. This increased sensitivity is largely attributable to the high metabolic rate of corneal epithelial cells, rendering them highly sensitive to chemical agents, and the large aqueous–mucous interface between the corneal surface and the tear film, which is a very effective sink for liquid and particulate contaminants as well as gas absorption (Pickard, 1919; Solberg et al., 1997; Safarinejad et al., 2001). Therefore, it is not surprising that approximately 75%–90% of HD casualties develop ocular symptoms, with 10% presenting with severe ocular damage (Pickard, 1919; Pechura and Rall, 1993). For example, of 998 victims exposed during the production of sulfur mustard at Edgewood Arsenal from September 1941 to March 1943, 78% developed acute ocular injuries (Uhde, 1946). A similar ratio held true for battlefield casualties during World War I and the Iran–Iraq War (Tuorinsky, 2008).

The spectrum of ocular injury among sulfur mustard victims is primarily the product of exposure time and concentration, with clinical outcomes ranging from mild conjunctivitis to chronic advanced corneal disease and loss of

FIGURE 36.4 *Gassed*, by John Singer Sargent. *Reprinted with permission from the John Singer Sargent Gallery.*

vision (Pechura and Rall, 1993). A remarkable series of low-dose HD exposure experiments involving live human military volunteers provided rigorous concentration:time (Ct) data for the acute phase of ocular mustard injury (Reed et al., 1918; Walker et al., 1928; Guild et al., 1941; Anderson, 1942; Smith and Dunn, 1991). Based on these experiments, ocular exposure to HD can be detected from exposure to 5–30 $mg \cdot min/m^3$. This dose produces a mild conjunctival injection without corneal involvement that resolves within days. Exposure to 60–75 $mg \cdot min/m^3$ HD causes conjunctivitis, corneal irritation, and photophobia (Mandel and Gibson, 1917; Mann and Pullinger, 1944; Gates and Moore, 1946; Balali-Mood and Hefazi, 2005). In some cases, exposure to 60 $mg \cdot min/m^3$ HD produces incapacitating ocular injuries that require supportive care for up to 2 weeks. Battlefield exposures and accidental exposure at ammunition plants provided additional data on outcomes at higher HD doses. At doses exceeding 100 $mg \cdot min/m^3$, severe intraocular injuries characterized by corneal edema, keratitis, ocular pain, blepharospasm, uveitis, neovascularization, and corneal perforation develop at greater than 100 $mg \cdot min/m^3$. The resulting corneal epithelial vesication and corneal edema require weeks to months to heal. Doses of 200 $mg \cdot min/m^3$ cause corneal edema, corneal opacity, eyelid edema and severe blepharospasm, and require months to heal. At doses exceeding 400 $mg \cdot min/m^3$, severe corneal damage develops that requires months of hospitalization. At these doses, blindness can rapidly develop due to corneal scarring and corneal ulceration. Based on these experiments, the minimum incapacitating vapor dose was estimated to be 60 $mg \cdot min/m^3$ and the median incapacitating dose (ICt_{50}) was estimated to be 100–200 $mg \cdot min/m^3$ (Project Coordination Staff, 1946; Reutter and Wade, 1994). Estimates of battlefield air concentrations of mustard gas during World War I were 19–33 mg/m^3. Under these conditions, the minimum incapacitating dose would be exceeded within 2 min, and a 5- to 10-min exposure would incapacitate 50% of soldiers (Solberg et al., 1997). Note that ocular exposure to neat HD in its liquid form, which is more concentrated than saturated HD vapor, dramatically increases the risk of corneal ulceration and vision loss, and therefore is far more dangerous than vapor-induced injury (Papirmeister et al., 1991).

Approximately 90% of soldiers and workers exposed to sulfur mustard in World War I initially presented with symptoms of conjunctivitis, photophobia, and blepharospasm. The subsequent injury progression was observed to follow one of three clinical trajectories (Classes I–III), which differed based on the nature of the symptoms and the recovery time (Table 36.3) (Hughes, 1945b). Class I injuries occurred in 75% of those with ocular presentation, involving mild symptoms without corneal involvement or significant chemosis that resolved within 1–2 weeks. Based on the Ct data presented here, Class I injuries are caused by doses ranging from 5 to 60 $mg \cdot min/m^3$. Class II injuries occurred in 15% of the victims, and correspond to a dose of 100–200 $mg \cdot min/m^3$. Class II injuries involve eyelid, conjunctival lesions and corneal lesions, and result in incapacitation lasting 4–6 weeks. Signs of the Class II injury include chemosis, corneal edema, epithelial erosions, severe ocular pain, blepharospasm, and photophobia. Class III injuries occurred in 10% of victims and are caused by exposure to more than 200 $mg \cdot min/m^3$. Class III injuries are characterized by severely affected eyes, with significant corneal involvement. Symptoms include severe ocular pain, reduced vision, blepharospasm, uveitis, edematous eyelids, limbal necrosis, chemosis, corneal erosions, and corneal edema. Class III was further subdivided into Class IIIa and Class IIIb. Class IIIa victims presented with moderate corneal symptoms, with a prognosis of 6 weeks to 3 months before soldiers could return to duty. In contrast, Class IIIb victims exhibited severe corneal changes resulting in

TABLE 36.3 Summary of ocular manifestations and healing times following exposure to SM vapor.

Injury class	Estimated dose ($mg \cdot min/m^3$)	Characteristic ocular manifestations	Symptomatic recovery
Class I	12–70	Conjunctival injection and ocular irritation, without lacrimation, blepharospasm, or photophobia	1–2 weeks
Class II	100–200	Above symptoms plus mild corneal involvement, such as corneal edema and epithelial vesication. Symptoms appear 6–12 h after exposure, including edema and epithelial erosions	6–12 weeks
Class III	>200	Corneal swelling, edema, and epithelial lesions, with destruction of limbal blood vessels at higher doses. MGK may develop. At very high doses, penetrating corneal ulcers occur	Months to never

Source: Adapted from Solberg, Y., Alcalay, M., Belkin, M., 1997. Ocular injury by mustard gas. Surv. Ophthalmol. 41 (6), 461–466.

disability of more than 3 months, and possibly causing complete loss of vision. Of the 939 ocular casualties caused by accidental exposures in mustard gas production factories in England, 10.4% presented with Class III symptoms, but only 1% developed Class IIIb corneal lesions (Hughes, 1945a).

36.4.1.2 Evidence for a delayed ocular mustard injury in human victims

Starting in the late 1920s, reports appeared of a late-onset complication in survivors of severe HD ocular injuries, in which ostensibly healed eyes developed a "delayed keratitis" from 8 to 25 years after exposure (Pechura and Rall, 1993). It was initially noted that following recovery from the acute lesion, which took 4–6 months, some patients remained largely asymptomatic for a decade or longer. However, approximately 1% of survivors subsequently developed a progressive ulcerative corneal disease that did not heal, characterized by photophobia, lacrimation, recurrent corneal erosions, idiotypic keratitis, decreased corneal sensitivity, and progressive corneal degeneration. As of 1939, approximately 300 victims of the severe form of mustard gas keratopathy (MGK) were described in the literature (Phillips, 1940). In 1944, it was reported that MGK developed intermittently in Class III-injured veterans over a period of 8–17 years after initial exposure, with a sudden increase in incidence from 17 to 25 years (Mann, 1944). Strikingly, of the 84 patients with delayed-onset HD injuries that were involved in that study, 76 were believed to have fully recovered from the acute injury prior to clinical re-emergence.

Additional evidence for the late-onset injury accumulated among survivors of the Iran–Iraq War, which represented the first instance of the prolonged deployment of HD munitions during the modern era, producing 50,000–100,000 military and civilian casualties (Willems, 1989; Khateri et al., 2003). Medical follow-up of this population using modern clinical methods has been illuminating with regard to the scope and progression of the acute and late injuries. In the largest available study, 34,000 survivors of HD injury were screened 13–20 years after exposure (Khateri et al., 2003). While 60.7% of survivors had no ocular symptoms, 35% were classified as having mild symptoms, involving persistent conjunctival irritation; 3.6% as having moderate symptoms, involving corneal opacities, mild corneal edema, and band keratopathy; and 0.7% as having severe symptoms, which included corneal melting and neovascularization. In a separate, nonoverlapping study involving 134 patients examined 17–22 years after exposure, 83% of survivors presented with ocular complications, which included burning (69%), photophobia (64%), blepharitis (28%), tearing (12%), corneal ulceration (12%), and retinal and conjunctival complications (4.5%) (Namazi et al., 2009). In another study specifically evaluating ocular injury in 40 veterans from 16 to 20 years after a single, high-dose exposure, 39 reported persistent ocular sequelae, including chronic conjunctivitis (17.5%), corneal thinning (15%), limbal ischemia (12.5%), corneal opacity (10%), corneal vascularization (7.5%), and corneal epithelial defects (5%) (Balali-Mood et al., 2005). A total of six patients exhibited severe corneal involvement and were diagnosed with the severe form of MGK.

This delayed ocular HD injury is predominantly associated with Class III exposures and exhibits latencies ranging from 1 to 40 years (Solberg et al., 1997; Balali-Mood and Hefazi, 2005). Onset is typically abrupt, characterized by the appearance of photophobia, tearing, and corneal and limbal lesions. However, the etiology of the late HD injury is still unknown. Corneal tissues removed from MGK victims during surgical interventions or postmortem exhibit signs of chronic inflammation, corneal thinning and ulceration, neovascularization, and corneal degeneration, suggesting a persistent injury that is beyond the healing capacity of the cornea (Richter et al., 2006; Javadi et al., 2007; Kanavi et al., 2010). Without a clear understanding of the etiopathogenesis of chronic HD injury, treatments have been mostly palliative in nature. Surgical interventions such as corneal keratoplasty and limbal stem cell transplants have had mixed outcomes, with successes largely restricted to mild cases of MGK (Richter et al., 2006; Javadi et al., 2007, 2011; Baradaran-Rafii et al., 2013).

36.4.1.3 Toxicokinetics of the acute and late-onset ocular mustard injuries

The acute symptomatic progression of the ocular HD injury is well described (Mann and Pullinger, 1944; Tuorinsky, 2008). The duration of the latent period between exposure and the appearance of ocular clinical symptoms is inversely correlated to the severity of exposure (Tuorinsky, 2008). At vapor doses below 60 $mg \cdot min/m^3$, the latent period is typically 4–12 h. At vapor doses exceeding 60 $mg \cdot min/m^3$, the latent period may be 3 h or less. Symptoms typically appear within an hour after exposure to liquid sulfur mustard. Following the latent period, the earliest symptoms of injury are sensations of grit in the eyes, ocular soreness, lacrimation, conjunctival injection, chemosis, and corneal injection. In Class II or III injuries, the corneal epithelium sloughs from the basement membrane within 6–12 h, leading to severe pain, photophobia, corneal edema, and impaired vision. By 24 h, corneal edema has increased the thickness of the cornea up to 300%, disrupting vision and

causing corneal pain. After a week, corneas begin to show clinical improvement with subsiding edema. Class II injuries subsequently develop superficial corneal vascularization, secondary corneal edema, and recurrent corneal symptoms that persist for several weeks before ultimately resolving.

The progression of healing in more severely injured eyes is less straightforward. A review of dose−response data and case studies enabled clinicians to identify three clinical trajectories among Class III-injured eyes: (1) injury resolution similar to Class II, but over a period of months and without the subsequent reoccurrence of corneal symptoms; (2) a chronic injury that develops immediately after the acute injury and fails to heal; and (3) delayed-onset lesions that appear 1−40 years after exposure (Duke-Elder, 1972; Papirmeister et al., 1991; Javadi et al., 2005, 2007). The latter two trajectories are collectively referred to as MGK. Although the relationship between the chronic and delayed-onset forms of MGK is unclear, the severe form of each involves an idiotypic, noninfectious keratitis with secondary keratopathies such as persistent epithelial lesions, corneal neovascularization, and progressive corneal degeneration (Khateri et al., 2003; Mousavi et al., 2009). Since both the chronic and delayed-onset forms of MGK result from a severe corneal exposure and share similar symptoms, it may be that a common pathophysiological mechanism is involved, despite temporal differences in clinical onset.

The pathogenic mechanisms responsible for MGK are unknown, but the most common clinical sequelae include recurring corneal epithelial lesions, corneal neovascularization, a progressive corneal degeneration, and frequent impairment or loss of vision (Solberg et al., 1997; Javadi et al., 2005; Balali-Mood and Hefazi, 2006). In a study restricted to 48 veterans suffering chronic ocular symptoms, 31 (64.6%) developed MGK directly from acute injury (i.e., the chronic form), whereas the delayed-onset form developed in 17 (35.4%), with latencies ranging from 1 to 15 years (Javadi et al., 2005). Limbal lesions were present in 81% of the MGK eyes, suggesting that development of limbal stem cell disorder is a common sequelae of MGK. Corneal signs included scarring (87.5%), neovascularization (70.8%), thinning (58.3%; resulting in corneal perforation in 4.2% of patients), and recurring epithelial defects (31.3%). In a separate study involving 149 seriously eye-wounded veterans, 90% of casualties with chronic ocular symptoms who were followed for 10−15 years showed no improvement, and in many cases, the injury became progressively worse (Ghasemi et al., 2009). According to these studies and many others, the delayed HD injury is usually progressive and difficult to clinically manage (Safarinejad et al., 2001). Not surprisingly, chemical warfare survivors with ophthalmologic complications suffer from a significantly lower quality of life as a consequence of their chronic injuries (Mousavi et al., 2009).

36.4.1.4 Mechanistic studies of sulfur mustard toxicity

Animal models have been used to study the pathogenesis of acute and chronic HD ocular injuries for over 70 years (Mann and Pullinger, 1944; Pechura and Rall, 1993; Ruff et al., 2013). One of the most productive models is an in vivo vapor exposure model in rabbits. The rabbit cornea is structurally similar to that of the human, and at functionally equivalent doses, rabbit and human eyes exhibit nearly identical lesions (Mann and Pullinger, 1944; Gates and Moore, 1946). Recently, a rabbit vapor HD exposure model was shown to produce dose-dependent, multiphasic corneal injuries with sequelae similar to those observed in human victims (Kadar et al., 2001; Milhorn et al., 2010; McNutt et al., 2012a,b).

Using the rabbit vapor exposure model, the progression of structural, biochemical, and molecular changes during acute injury and the transition to MGK were elucidated. Briefly, exposed corneas develop an acute lesion within 1 day, characterized by vesication of the corneal epithelium, stromal keratocytosis, corneal edema, and CEC loss. The corneal epithelium then undergoes a robust healing response, regenerating an intact, stratified epithelium with rudimentary hemidesmosomal attachments to adhesion plaques in the anterior stroma by 7 days after the exposure. Grossly, corneas appear to be healing from 1 to 2 weeks (the quiescent phase), with few clinical symptoms other than lingering edema. Resolving corneas subsequently undergo a rapid decrease in corneal thickness, reaching baseline levels by 6 weeks; a time frame that is similar to Class II or IIIa injuries in humans (Fig. 36.5) (Kadar et al., 2001; McNutt et al., 2012a,b). In contrast, the failure of corneal edema to resolve at 3 weeks and beyond is the earliest clinical marker of MGK onset. Histopathological markers of MGK include recurring basal epithelial cell cytotoxicity, basement membrane zone degeneration, loss of LESCs, inflammatory cell infiltration, elevation of cytokines, persistent endothelial failure, delayed in-migration of keratocytes, stromal degeneration, and redundant deposition of basement membrane components caused by cyclical attempts to regenerate the epithelium (Kadar et al., 2001; McNutt et al., 2012a,b). Secondary pathologies, such as epithelial bullae formation, recurring corneal erosions, neovascularization, and LSCD, subsequently develop, further interfering with stable repair of the ocular surface. Postexposure, steroid-based antiinflammatory protocols reduce and postpone the appearance of late injury. However, they do not prevent late injury, suggesting that anti-inflammatory drugs fail to treat the central pathologies responsible for MGK onset (Amir et al., 2000; Kadar et al., 2009; Gordon et al., 2010).

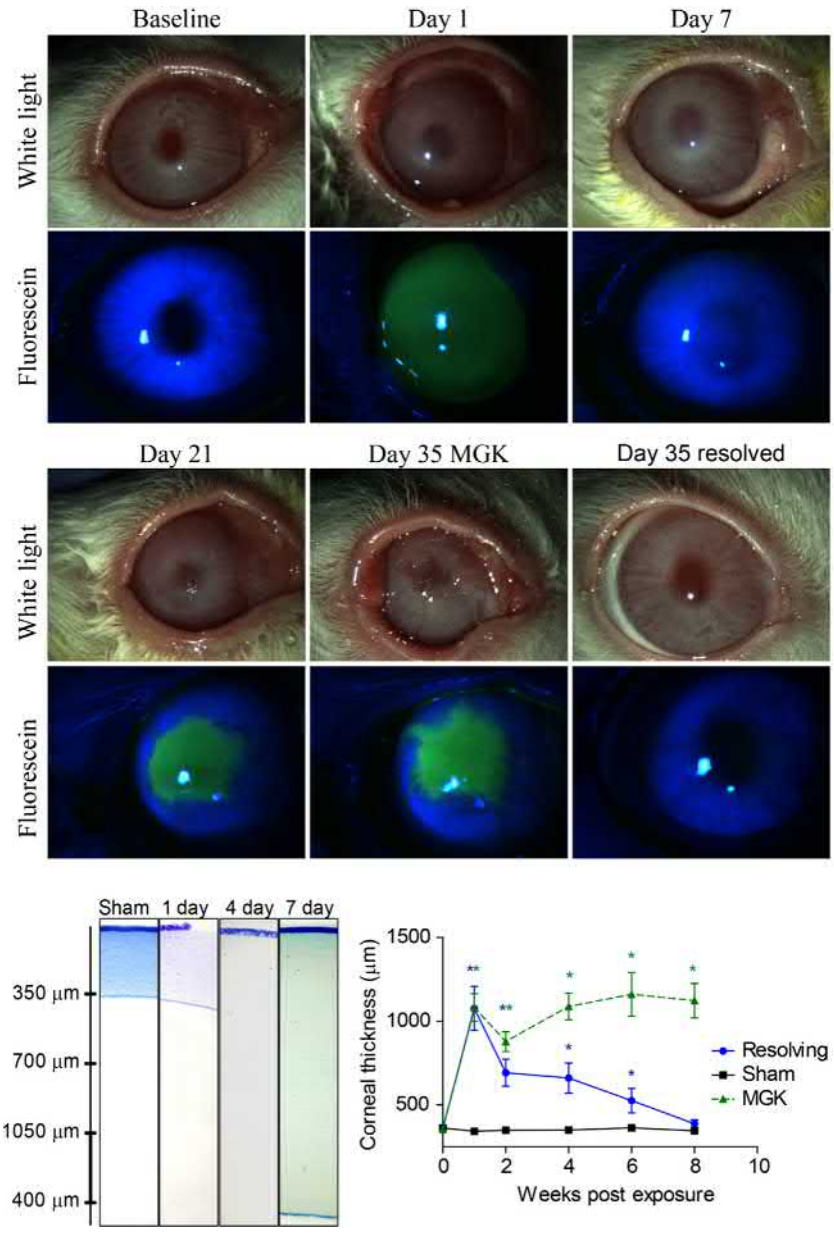


FIGURE 36.5 Development of acute and MGK injuries in HD vapor-exposed rabbit eyes.
(Top panels) Rabbit eyes were exposed to HD vapor for 2.5 min and evaluated by slit-lamp examination using fluorescein uptake as a measure of epithelial vesication. All panels are from the same animal. Note the transient recovery of an impermeable epithelial cap by 7 days, only to be followed by a recurring epithelial lesion by 21 days in an MGK cornea. Middle right represents a resolved eye, which appeared to have fully healed. (Bottom left) Histology demonstrating massive increase in corneal edema during the first week, a result of cytotoxicity in the corneal epithelium and endothelium. Note: lesion margin in the epithelium of the 1 d cornea. (Bottom right) Longitudinal changes in corneal edema in resolving versus MGK eyes. Note the biphasic transition at 2 weeks in MGK corneas.

The distinct pathophysiology of MGK revealed in these studies suggests the involvement of injury processes that operate on different time scales and in different corneal compartments than during the acute injury. However, all studies identified persistent edema as a primary causative factor underlying MGK onset.

36.4.1.5 Etiogenesis of the delayed ocular sulfur mustard injury: current theories

Damages to the corneal limbus and the corneal endothelium have emerged as pathophysiologies that may be critically involved in MGK onset, progression, or both. Both pathologies are consistent with clinical observations in human MGK corneas and are spatiotemporally expressed in such a way as to contribute to the long-term corneal HD injury (Baradaran-Rafii et al., 2010; Jafarinasab et al., 2010).

In a clinical study of 35 Iranian patients diagnosed with MGK, all MGK eyes exhibited LSCD (Baradaran-Rafii et al., 2010). Similar symptoms were observed in rabbit eyes expressing MGK symptoms, including goblet cell invasion (an indicator of limbal destruction) and a significant reduction of LESCs (Kadar et al., 2011, 2012). Interestingly, LESC loss did not appear to occur during

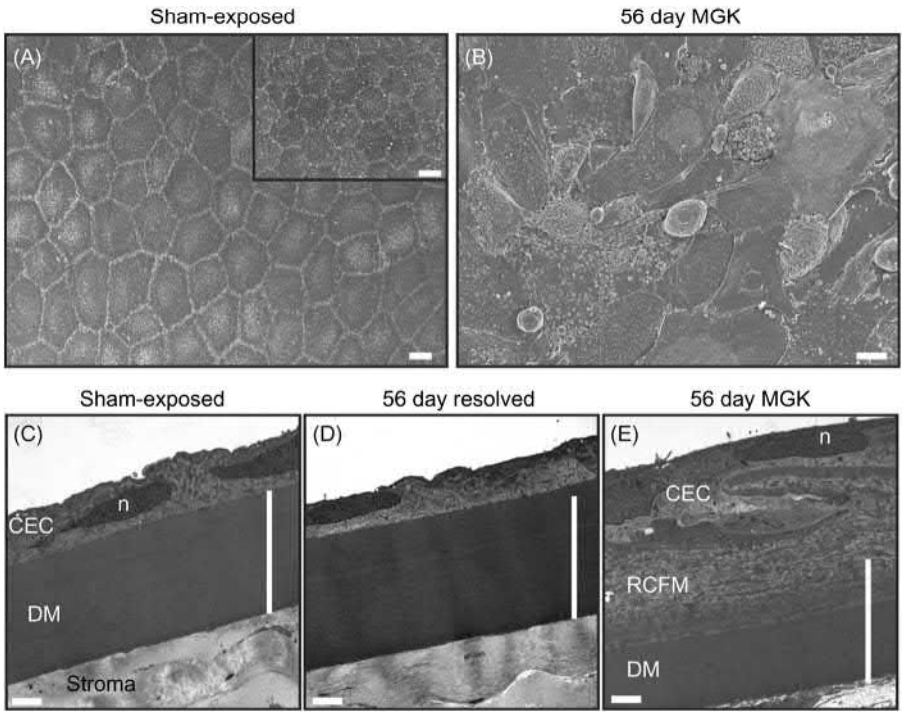


FIGURE 36.6 Ultrastructural evidence of endothelial cytotoxicity.
(A and B): Scanning micrographs showing CEC morphologies in resolved (A) and MGK corneas (B−D) 8 weeks after SM exposure. Inset in (A) is a sham-exposed cornea at the same magnification, highlighting the increased size of CECs in resolved corneas. Note evidence of ongoing cytotoxicity and a delayed healing response in (B). Scale bars = 10 μm. Transmission micrographs of posterior cornea comparing ultrastructure of sham-exposed (C), resolved (D), and MGK (E) corneas 8 weeks after SM exposure. Note the extensive thickening of the posterior DM and formation of a retrocorneal fibrous membrane in the MGK cornea. For comparison, the vertical white lines represent the full thickness of the DM in sham-exposed controls. Labels: corneal endothelial cell (CEC); nucleus (n); Descemet's membrane (DM); retrocorneal fibrous membrane (RCFM). Scale bars = 2 μm.

the acute phase following HD exposure; rather, it developed concurrent with the appearance of MGK symptoms. These data suggest that dysfunction of the LESC niche is a delayed phenomenon that occurs after the acute injury. A proinflammatory response and nerve damage were found in the corneal limbus of both rabbits and humans during MGK onset, implicating their involvement in the initiation of the late ocular injury (Javadi et al., 2005; Kanavi et al., 2010; Kadar et al., 2011). Regardless of the causal relationship between LSCD and MGK, LSCD is a clinically important aspect of MGK and the delayed loss of LESCs raises the possibility of a postexposure therapeutic window during which limbotrophic treatments could preserve LESCs.

A second important pathology that was recently described is failure of the corneal endothelial barrier due to CEC loss. Early reports of HD toxicity included endothelial cell toxicity and uveitis in Class III injuries, suggesting that HD can penetrate through the cornea to the anterior chamber (Mann and Pullinger, 1944; Hughes, 1945b). Evidence of endothelial toxicity was identified in human MGK corneas, including reduced CEC density with increased variability in size and morphology (Jafarinasab et al., 2010). More recently techniques such as transmission electron microscopy, in vivo confocal microscopy, functional assessment of the barrier, and immunocytochemistry were used to demonstrate that corneal endothelial toxicity is associated with acute injury and MGK corneas, but not resolved corneas (McNutt et al., 2013). MGK corneas exhibited an idiotypic endothelial injury characterized by focal CEC loss, abnormal CEC morphologies, and a diffusively thickened DM (Fig. 36.6). In contrast, resolved corneas exhibited a normal-appearing corneal endothelium. Notably, other clinical endotheliopathies involving the rapid loss of large quantities of CECs, such as aphakic bullous keratopathy, produce secondary keratopathies that are strikingly similar to MGK, including epithelial bullae, delayed LSCD, and corneal inflammation (Taylor et al., 1983; Eagle et al., 1989; Alomar et al., 2011).

The potential involvement of endothelial toxicity in the late HD injury is intriguing for several reasons. First, it provides a mechanism to explicate the dose dependence of MGK onset, based on the permeation of sufficient doses of HD through the cornea to cause injury to the corneal endothelium. Second, it proposes that the clinically biphasic injury progression observed during the acute phase is an epiphenomenon arising from the differential healing capacities of the corneal epithelium and corneal endothelium. Furthermore, the limited ability to heal large endothelial lesions may contribute to the prolonged recovery time following Class III ocular injuries. Third, it provides a single pathology to explain both the chronic and delayed-onset forms of MGK. For example, in this model, the chronic form of MGK would be caused by the inability to restore an intact endothelial barrier, resulting in untreatable corneal edema. Alternatively, in the delayed-onset form of MGK, the delayed loss of CECs (i.e., as a consequence of aging, or due to delayed cytotoxic mechanisms such as genotoxicity) from an endothelium

that is already diminished due to HD injury eventually results in endothelial failure and corneal decompensation. Additional implications of this hypothesis include the possibilities that therapies to restore the endothelium may mitigate the likelihood of MGK and that quantitation of endothelial loss may provide a diagnostic to identify corneas likely to develop MGK. Although many questions remain regarding the extent to which endothelial cytotoxicity and LSCD contribute to the acute injury and MGK, it is encouraging that mechanistic studies have moved beyond the corneal epithelium to study HD-induced injury processes in other corneal tissues.

36.4.2 Lewisite

Lewisite (L; $C_2H_2AsCl_3$) was first synthesized in 1904 and stockpiled by the United States, Germany, and Japan during World War II. Although lewisite is considerably more potent than HD, it produces immediate symptoms of physical discomfort, which rendered it relatively ineffective as a standalone CWA. However, lewisite was found to increase the environmental persistence of HD by depressing its freezing point at lewisite concentrations that had no apparent physiological effect, and therefore lewisite stockpiles were retained for several decades.

The toxicological effects of ocular exposure to lewisite are mediated by the interaction of inorganic arsenite (AsO_3^{3-}) with thiol groups of biologically active proteins, including dihydrolipoic acid (DHA). DHA is a cofactor in several critical enzyme systems critically involved in energy production, and disruption of DHA inactivates these enzyme complexes. For example, As^{3+} interaction with the E3 component of the pyruvate dehydrogenase complex prevents the conversion of pyruvate to acetyl-CoA, impairing ATP production and resulting in energy depletion, metabolic failure, and cytotoxicity (Young, 1999). Though lewisite injury progression can be mitigated by treatment with sulfhydryl-containing competitive antagonists of lewisite, such as 2,3-dimercaptopropanal (aka, British antilewisite; BAL), an ocular formulation of BAL is not currently available (Vilensky and Redman, 2003). Ocular neutralization of lewisite is very time-critical; unless BAL is topically administered with 2–5 min, lewisite eye injuries are irreversible.

36.4.2.1 Toxicokinetics of ocular lewisite injuries

The distinctive toxicokinetic effects of lewisite exposure on ocular tissues produces an injury progression that is very different from the mustards (Ottinger et al., 1973; Romano et al., 2008; Tuorinsky, 2008). Unlike the latency associated with mustard exposure, lewisite causes rapid ocular irritation, lacrimation, blepharospasm, and chemosis. Eye pain occurs immediately, reaching its peak in 4–8 h. Corneal vesication, perforation, and blindness are observed following instillation of as little as 1 μL of neat lewisite to the corneal surface (Pechura and Rall, 1993). Vapor administration of 0.15 $mg \cdot min/m^3$ causes significant conjunctival injection and swelling of the eyelids, whereas corneal injuries occur at concentrations as low as 2.5 $mg \cdot min/m^3$. Permanent eye damage occurs at concentrations as low as 15.2 $mg \cdot min/m^3$. Lewisite can be detected by smell at about 14–23 mg/m^3; thus, permanent ocular injury is likely within 1 min of olfactory detection (Gates et al., 1946). At high vapor doses or following liquid droplet exposure, delayed effects develop in the cornea within 6–24 h, including vesication of the corneal endothelium, full-thickness keratocytosis, corneal edema, inflammatory cell infiltration, neovascularization, corneal perforation, and blindness.

Despite the 20- to 30-fold higher toxicity of lewisite than mustards, the ocular injuries caused by field exposure to lewisite vapor are predicted to be less significant than HD (Gates et al., 1946). Because ocular irritation is almost immediate even at low concentrations of lewisite, the rapid onset of blepharospasm, ocular pain, and edema at low concentrations causes the eyes to close involuntarily, reducing the total ocular exposure.

Lewisite is very lipophilic, and ocular absorption has been reported to elicit toxicological responses in the ciliary body and iris (Young, 1999). At similar doses, it is significantly more efficient than HD at evoking corneal edema (Pechura and Rall, 1993). At high doses, lewisite-exposed eyes rapidly develop miosis and severe uveitis, indicating that lewisite penetrates to the inner chambers of the eye (Pechura and Rall, 1993). Since lewisite efficiently permeates the cornea to produce acute toxicity in posterior ocular tissues, it is also likely to cause CEC toxicity. This is consistent with a 1947 report that first described acute symptomatic similarities between the mustards and lewisite, despite their distinctive modes of actions (Adler et al., 1947). Notably, this study also reported the focal loss of CECs following liquid instillation of lewisite directly to the corneas of rabbits. It is currently unknown if lewisite exposure produces the equivalent of a late HD injury. While delayed-onset ocular effects analogous to severe HD-exposed eyes were not reported in lewisite-exposed rabbits, rabbits were only followed for 30 days after exposure (Mann et al., 1946).

36.4.3 Phosgene oxime

Although technically considered a vesicant, phosgene oxime (CX; Cl_2CNOH) does not produce blisters, and thus it is more appropriately considered an urticant, or nettle agent. Phosgene causes significant pain on exposed skin and eyes through an unknown mechanism. Eyes rapidly develop conjunctivitis, lacrimation, lid edema, and

blepharospasm after mild exposures, while more severe exposures can result in keratitis, iritis, corneal perforation, and blindness (Romano et al., 2008). In general, eye lesions are fairly similar to those caused by lewisite. Phosgene oxime ocular injury has not been well studied, and long-term consequences of exposures are unknown.

Inhalational injury studies conducted in humans are limited to a controlled study with informed volunteers (Malatesta et al., 1983). This study reported that a vapor concentration of 1 mg/m^3 was the limit of physiological detection. Exposure to 3 $mg \cdot min/m^3$ was defined as the minimum effective concentration, eliciting conjunctival irritation. In the same study, mice, guinea pigs, and rabbits exposed to 3000−15,000 $mg \cdot min/m^3$ phosgene oxime displayed agitation, respiratory difficulty, and intense lacrimation within 30 min. These symptoms appeared to resolve within 72 h, although no supportive histology or histopathology data were published.

36.5 Nerve agents

Schedule 1A OPNAs include the G-series agents, the V-series agents, and novichok agents (Table 36.1). OPNAs inactivate AChE by alkyl phosphorylation of a serine hydroxyl group at the esteratic site of the enzyme (Romano et al., 2008). Once conjugated to AChE, the nerve agent eventually loses an alkyl side chain by hydrolysis in a process known as *aging*, further enhancing the stability of the enzyme−nerve agent complex. Whereas reactivators such as the quaternary ammonium oxime family can restore function to nonaged AChE, there is currently no way to reactivate aged AChE in vivo, and thus recovery of cholinergic function mainly depends on synthesis of new enzyme. Functionally, OPNA inhibition of AChE prolongs the residency of the ACh neurotransmitter in the synapse, resulting in the excessive stimulation of postsynaptic receptors. In the peripheral nervous system, toxidromic progression is mediated by cholinergic overstimulation of several types of receptors, including nicotinic receptors at neuromuscular junctions; nicotinic receptors at autonomic ganglia; muscarinic receptors at parasympathetic efferents onto smooth muscles; and muscarinic receptors on adrenal glands and sweat glands. Unlike the severe pathologies resulting from ocular exposure to the cytotoxic ocular irritants, the neuro-ophthalmic effects of exposure to OPNAs are fully reversible.

Ocular symptoms are among the earliest and most sensitive indications of the cholinergic toxidrome caused by exposure to nerve agent vapor, including tearing, pupillary constriction (miosis), and loss of accommodation. Ocular symptoms of a cholinergic toxidrome can manifest at vapor doses that do not cause evidence of other toxicities, and therefore can serve as an early physiological marker of exposure to nerve agents (Baker and Sedgwick, 1996). For example, the most reliable indicator of exposure after the Tokyo sarin attack was miosis, which was observed in 90% of victims (Wiener and Hoffman, 2004). Ocular toxicity by OPNAs is specifically mediated by overstimulation of muscarinic receptors and excessive excitation of parasympathetic signaling within the eye, causing contraction of the pupillary sphincter and ciliary muscle and overstimulation of the lacrimal glands, resulting in the clinical symptoms of miosis, blurred vision, and tearing (Romano et al., 2008).

The first intraocular effect of nerve agents is miosis resulting from overstimulation of parasympathetic muscarinic receptors on the pupillary sphincter muscle. This causes the iris to contract and reduces light input into the eye (see Fig. 36.2) (Soli et al., 1980; Dabisch et al., 2005). The second intraocular target of nerve agents is the ciliary muscles that control lens accommodation. Overstimulation of the ciliary muscles alters the lens shape, decreasing focal accommodation and blurring vision. Overstimulation of cholinergic nerve terminals in the retina may also contribute to visual disruption (Voigt, 1986). The final ocular targets of OPNA exposure are the parasympathetic inputs to the lacrimal glands, increasing tearing.

The miotic effect of OPNAs is primarily attributed to ocular absorption rather than systemic distribution, because percutaneous exposure does not cause miosis until near-lethal doses (Table 36.4; Romano et al., 2008). In contrast, OPNA exposure on or near the eye will cause miosis at very low concentrations (Hurst, 2007). Together, these suggest that the relatively privileged environment of the inner eye is more susceptible to direct absorption of nerve agent and drugs than to systemically circulated compounds. Furthermore, they indicate that the

TABLE 36.4 Comparison of OPNA lethal Ct_{50} and miosis Ct_{50} values.

Agent	Lethal Ct_{50} ($mg \cdot min/m^3$)	Miotic Ct_{50} ($mg \cdot min/m^3$)
GA	400	2−3
GB	100	3
GD	70	<1
GF	Unknown	<1
VX	50	0.04

Source: Modified from Hurst, G., US Army Medical Research Institute of Chemical Defense, Chemical Casualty Care Division, 2007. Chemical Casualty Care Division's Field Management of Chemical Casualties Handbook. Chemical Casualty Care Division; US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD and Romano, J.A., Lukey, B.J., Salem, H., 2008. Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology, and Therapeutics. CRC Press, Boca Raton, FL.

onset of tearing with miosis, in the absence of pain, is a prodromic indicator of exposure to OPNAs.

36.6 Psychomimetic incapacitating agents

Incapacitating agents are defined by the Department of Defense as "an agent that produces temporary physiological or mental effects, or both, which will render individuals incapable of concerted effort in the performance of their assigned duties" and are not intended to be lethal (Romano et al., 2008). The development and use of psychomimetics as incapacitating agents are reflected in diverse apocryphal stories, driven in part by the testing of lysergic acid diethylamide (LSD), phencyclidine (PCP), and 3-quinuclidinyl benzilate (BZ) on military volunteers at Aberdeen Proving Ground (McFarling, 1980; National Research Council, 1984). Collectively, the incapacitating agents are an unusual category of CWAs, with varied physicochemical characteristics and psychogenic effects. Incapacitating agents involve a spectrum of psychogenic effects, including agents that act as stimulants, depressants, psychedelics, and deliriants. Psychomimetic agents that have been specifically described in a military context include muscarinic antagonists, cannabinoids, indoles, and anxiogenics.

The only psychomimetic chemical explicitly listed in the CWC is BZ, a Schedule 2A agent. BZ is an anticholinergic glycolate related to atropine that competitively antagonizes postjunctional muscarinic receptors at ganglia as well as parasympathetic innervations onto smooth muscle and exocrine glands. By blocking the ability of muscarinic receptors to respond to the synaptic release of acetylcholine, BZ inhibits parasympathetic signaling and drives the ocular nerves toward sympathetic dominance. This shifts the pupillary balance toward mydriasis, failure of accommodation, and lacrimal paralysis. Thus, BZ effects on the eye are the opposite of the OPNAs, resulting in mydriasis, the loss of near-focus, and dry eye. Similar to nerve agents, once normal cholinergic signaling is restored, the ocular effects of BZ are fully reversed.

Unlike the prodromic effects of many agents on the ocular system, ocular symptoms of BZ inhalation occur secondary to more general signs of intoxication, such as incoordination, confusion, and slurred speech (Sidell et al., 1997). Although minimal human dose–response data is available for ocular symptoms of BZ exposure, relevant studies were conducted in other species. The effective species-specific concentration:time values for mydriasis following inhalational exposure of BZ were less than 130 $mg \cdot min/m^3$ in dogs, 70 $mg \cdot min/m^3$ in monkeys, and 40 $mg \cdot min/m^3$ in rabbits (Ketchum, 1963; McNamara, 1963). At incapacitating doses (estimated to be 8 μg/kg), central effects persist in humans for 3–4 days; however, the persistence of ocular effects after such treatments was not described.

36.7 Blood agents

The principal blood agents are hydrogen cyanide (AC; HCN) and cyanogen chloride (CK; NCCl). These agents are Schedule 3 toxic chemicals according to the CWC. Schedule 3A substances have legitimate, large-scale industrial uses; therefore, large stockpiles are likely available for repurposing as CWAs.

Cyanide is one of the least toxic of the lethal CWAs. The inhalational LCt_{50} values for AC and CK are estimated to be 2500–5000 and 11,000 $mg \cdot min/m^3$, respectively (Simeonova, 2004). The cyanide ion (CN^-) is the toxic moiety, mediated primarily by its great affinity for the heme a_3 moiety of cytochrome c-oxidase in mitochondria, a key component in oxidative respiration. This interaction blocks the last stage in the electron transfer chain, resulting in cellular hypoxia and a shift of aerobic to anaerobic cellular respiration, and in turn causing cellular ATP depletion and lactic acidosis. Therefore, tissues with high metabolic demands, such as neurons and cardiac cells, are key targets for toxicity. At lethal doses, death occurs within 6–8 min (Sidell et al., 1997).

Exposure to blood agents has two distinct effects on the eye, depending on the route of exposure. The retina and optic nerve are principal symptomatic targets of acute systemic cyanide exposure, with mydriasis commonly occurring at sublethal cyanide exposure and vision failure developing at higher doses. This is due to the metabolic inhibition of highly active neurons secondary to vascular distribution of the CN^- ion. The appearance of fixed and dilated pupils is common late in the toxidromic progression, but this is likely to result from the general loss of autonomic neuronal function rather than ocular-specific toxicity (Grant and Schuman, 1993).

Alternatively, topical administration of blood agents to the ocular surface results in local absorption and toxicity, primarily concentrated in anterior ocular tissues such as the conjunctiva and lacrimal glands. Thus, topical exposure to the blood agents elicits mild ocular symptoms, primarily characterized by lacrimation and conjunctival irritation. In animal studies, the administration of sodium cyanide (1.7–5.3 mg/kg/day) to the inferior conjunctival sacs of rabbits resulted in immediate conjunctival irritation and lacrimation (Ballantyne, 1983b). In a separate study, rabbits that were administered 0.9 mg/kg of hydrogen cyanide to the conjunctival sacs developed general keratitis (Ballantyne, 1983a). These two experiments involved the administration of lethal doses of cyanide in liquid form directly to the conjunctiva, which is likely to lead to the very rapid cytotoxicity of local nerves, blood

vessels, and epithelial cells. Exposure to sublethal concentrations of cyanogen chloride vapor similarly caused intense conjunctival irritation, severe blepharospasm, and lacrimation.

36.8 Choking agents

The choking agents are chloropicrin (PS; CCl_3NO_2), phosgene (CG; $COCl_2$), and diphosgene (DP; $ClCO_2CCl_3$). These agents injure the nose, throat, and lungs, causing pulmonary edema and respiratory distress (Dembek, 2007). Although diphosgene has very similar physiochemical properties to phosgene, it readily condenses to a liquid and is therefore easier to handle and store. The ocular irritation caused by all three agents occurs at doses that are equivalent to the limit of olfactory detection. The acute symptoms of ocular exposure to phosgene and diphosgene (and possibly chloropicrin) are due to their hydrolysis to hydrogen chloride (HCl), creating what is essentially a chemical burn (Dembek, 2007). The longer term respiratory symptoms are primarily due to hydrolysis as well as acylation, resulting from reaction of phosgene with nucleophilic moieties on macromolecules, such as amino, hydroxyl, and thiol groups. Acylation denatures lipid and protein and disrupts enzymatic function and appears to play a more significant role than hydrolysis in longer term pathophysiologies. In contrast, the mechanism of action for chloropicrin is not well understood, but may involve an oxidative reaction with biological thiols, such as glutathione and hemoglobin.

Although there is a paucity of acute human data containing ocular pathogenesis and toxicokinetics caused by phosgene or diphosgene, ocular irritation involving burning, conjunctivitis, and lacrimation was reporteded within 20 min of exposure to 12 mg/m^3 phosgene (Bast and Bress, 2002). Concentrations of 30−40 mg/m^3 result in severe ocular irritation. Although phosgene's poor water solubility means that minimal hydrogen chloride is produced by hydrolysis, even small quantities are sufficient to elicit an initial irritation to the eyes, nasopharynx, and respiratory tract. Contact with liquid phosgene may result in corneal opacities and delayed perforation, consistent with a chemical burn injury and presumably resulting from the effects of HCl on the cornea.

Human exposure concentrations and times are better described for chloropicrin, which is roughly one-third as lethal as phosgene with an estimated median lethal dose of approximately 20,000 $mg \cdot min/m^3$. At low concentrations chloropicrin causes profuse lacrimation and conjunctivitis. At high concentrations it is severely irritating to the lungs, eyes, and skin. Thus, the primary effects observed with short- and long-term exposure to chloropicrin are sensory and respiratory irritation. The most likely route of ocular exposure is conjunctival and corneal absorption. Ocular irritation occurs prior to other symptoms of exposure, including respiratory irritation, and manifests at doses that are below the limit of olfactory detection. Studies in human volunteers indicate that lacrimation and blepharospasm are apparent at 5.0 $mg \cdot min/m^3$, conjunctival irritation is apparent at 10−100 $mg \cdot min/m^3$, and intolerable ocular irritation develops at 500 $mg \cdot min/m^3$ (Fries and West, 1921; Prentiss and Fisher, 1937). These studies are consistent with more recent findings following an industrial accident in California that produced a plume of chloropicrin with estimated air concentrations between 0.17 and 1.0 mg/m^3 (Barry et al., 2010). Of the 324 residents who reported symptoms consistent with chloropicrin exposure, 93.2% presented with ocular symptoms such as lacrimation, ocular pain, and burning.

36.9 Riot control agents

Riot control agents (RCAs), also called *irritants*, *lacrimators*, and *tear gas*, produce transient discomfort and eye closure that render the recipient temporarily incapable of fighting or resisting. The RCAs in use today are CS (2-chlorobenzalmalononitrile; $C_{10}H_5ClN_2$), CN (mace; C_8H_7ClO), CR (dibenzoxazepine; C_8H_7ClO), OC (oleoresin capsicum; $C_{18}H_{27}NO_3$), and various combinations of these agents. The RCAs share several characteristics that make them very effective as nonlethal agents, including rapid onset, with symptoms developing within seconds of exposure; a short duration of effect, with recovery occurring within 30 min after the end of exposure; and a high median lethal concentration with a low effective concentration meaning that they cause acute incapacitation without long-term effects or disabilities (Table 36.5) (Olajos et al., 2004). The eye is the most sensitive organ to RCAs, and although each RCA has slightly different effects, they all produce sensations of conjunctival and corneal burning, tearing, blepharospasm, and conjunctival injection. Barring direct application of solid RCAs to the eye or mechanical injury from the deployment of RCAs, the ocular symptoms are transient and fully reversible. Although RCAs are not considered to be CWAs according to the 1925 Geneva Convention, President Gerald Ford signed Executive Order 11850, banning the use of RCAs in war, except in defensive actions, without the advance approval of the president. Thus, although RCAs are incapacitating agents, they are not considered *military* incapacitating agents. However, since RCAs are increasingly being deployed in other contexts and can theoretically be deployed in a military capacity, we will address them here.

RCAs are distinguished based on two general mechanisms of action. The C agents (CS, CN, and CR) are SN_2-alkylating agents that react readily with sulfhydryl-containing enzymes,

TABLE 36.5 Toxicological parameters of RCAs.

Compound	Ocular irritancy	Rate of action	Irritancy threshold (mg/m^3)	Intolerable concentration (mg/m^3)	Lethal concentration ($mg \cdot min/m^3$)
CS	Profound	Instantaneous	0.004	5	25,000
CN	Profound	Instantaneous	0.3	35	14,000
CR	Profound	Instantaneous	0.002	1	100,000
OC	Profound	Instantaneous	–	–	–
Chloropicrin	High	Rapid	2–9	50	20,000

Source: Modified from Olajos, E.J., Woodhall, S., 2004. Riot control agents and acute sensory irritation. In: Olajos, E.J., Woodhall, S. (Eds.), Riot Control Agents and Acute Sensory Irritation. CRC Press LLC, Boca Raton, FL.

such as lactate dehydrogenase. For example, CS reacts rapidly with the disulfhydryl form of lipoic acid, a coenzyme in the pyruvate decarboxylase system. These agents act as peripheral sensory irritants, which act primarily upon the eyes, respiratory tract, and skin. Despite small differences in potency, the C agents are similar in that exposure results in the near-instant ocular irritation, burning, and swelling of the conjunctiva, with uncontrolled lacrimation, blepharospasm, and increased intraocular pressure. The use of CS has largely replaced CN due to being significantly more effective as a sensory irritant, but much less toxic. Ocular irritancy studies for the C agents were conducted in various animal species and in human volunteers (Punte et al., 1963; Ballantyne and Swanston, 1978). Generally, these studies were consistent in showing that exposure to low concentrations of CS was immediately irritating and rapidly became incapacitating at higher doses, but significant corneal toxicity did not occur until very high concentrations were achieved. For example, instillation of 5%–10% CS to the corneal surface caused acute conjunctivitis, chemosis, keratitis, neovascularization, epithelial lesions, and corneal inflammation. The mechanism of injury at high concentrations is unknown, although it is speculated that the alkylating properties of the C agents disrupt protein structure. The poor water solubility of C agents limits their principal mode of action to the corneal epithelium. The potential for ocular irritation from CS is less than with CN; however, prolonged exposures to both results in conjunctivitis and photophobia (Ballantyne et al., 1974).

The second class of RCAs is the capsaicins, represented by OC and its derivatives. The capsaicins are naturally occurring compounds of the capsicum plants, which include chili peppers and jalapenos. Capsaicin binds TRPV1, a vallinoid type I receptor that is activated by elevated temperature (43°C) or abrasion. TRPV1 is expressed by nociceptor neurons in the eye, and TRPV1 activation elicits symptoms of pain. Clinical signs of exposure to OC include lacrimation, transient conjunctivitis, redness, burning, pain, swelling, and blepharospasm. If applied directly to the eye, OC can cause neurogenic inflammation, insensitivity to chemical and mechanical stimuli, and loss of the blink reflex (Olajos and Salem, 2001). Generally, not much is known about the general toxicity of capsaicins, but because they are widely used in food products, they are believed to exhibit low ocular toxicities.

36.10 Biological toxins

Although there is ongoing disagreement over whether biotoxins should be classified as biological warfare agents (BWAs) or CWAs, biotoxins are clearly more similar to CWAs in their deployment, clinical manifestation, and medical management. Three categories of biotoxins are discussed below, each of which exhibits a distinctive mechanism of action: neurotoxins, metabolic toxins, and bacterial superantigens.

36.10.1 Botulinum neurotoxins (BoNTs)

BoNTs are highly lethal bacterial toxins produced by the *Clostridium* species, a family of bacteria that lives in the soil and in low-oxygen conditions. Clinical manifestations of botulism can occur following the ingestion, inhalation, or injection of preformed toxin or by productive infection of toxin-producing strains. There are currently eight known BoNT serotypes (/A–/H), of which /A, /B, /E, /F are commonly associated with human disease (Simpson, 2004; Dover et al., 2013). The toxin is expressed as a single 150-kDa peptide, which is post-translationally nicked to produce a dichain composed of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) linked by a disulfide bond (reviewed in Simpson, 2004). HC mediates binding to presynaptic receptors and entry of toxin into the neuron via synaptic endocytosis. Acidification of the endosome triggers the HC to form a pore in the endosome membrane that allows translocation of the LC from the lumen to the neuronal cytosol. LC then targets and cleaves one or more of three soluble NSF attachment

protein receptor (SNARE) proteins with exquisite specificity: synaptosomal-associated protein 25 (SNAP-25; BoNT/A, /C, /E); vesicle-associated membrane proteins 1−3 (VAMP1−3; BoNT-/B, /D, /F, /G); or syntaxin (BoNT/C). The neuronal SNARE proteins are essential components of the synaptic exocytosis mechanism, and their cleavage prevents functional assembly of the ternary complex, thereby blocking neurotransmitter release. The combination of efficient neuronal targeting and presynaptic activation renders BoNTs the most potent substances known, with estimated human median lethal doses as low as 0.1−1 ng/kg.

BoNTs interfere with the synaptic release of ACh from several types of cholinergic neurons, including motor neurons, preganglionic neurons, parasympathetic neurons, and some sympathetic neurons, such as those that innervate sweat glands. Furthermore, although there is a partial selectivity for cholinergic synapses, BoNT can also impair neurotransmission at other types of synapses, including adrenergic and noradrenergic sympathetic synapses (MacKenzie et al., 1982). Chemical denervation of autonomic signaling can occur at ganglia, which are cholinergic; at the synaptic fasciculation, where the neuron releases neurotransmitter onto the smooth muscle cells; or in both locations. Thus, the effects of intoxication on ocular behavior can vary based on dose and time after intoxication. Prominent neurologic findings in all forms of botulism include diplopia, blurred vision, and dilated pupils with weak pupillary responses. Tear production may be reduced because of peripheral parasympathetic cholinergic blockade, and ptosis is common.

The speed at which nerve terminals are intoxicated is closely related to neuronal activity, since higher rates of neurotransmitter release correspond to increased toxin uptake. Consequently, ophthalmic manifestations are among the earliest signs of clinical presentation (Konig et al., 1975; Levy et al., 1991). Common findings include accommodative paresis (59%), blurred vision (89%), mydriasis (52%), and photophobia due to paralysis of the cholinergic terminals of the parasympathetic nerves. Impairment of tear production results from paralysis of the autonomic nerves innervating the lacrimal glands, leading to dry eye. Paralysis of the extraocular muscles is also an early symptom, producing double vision (diplopia, 59%), fixed gaze (36%), nystagmus (56%), and drooping eyelids (blepharoptosis, 80%). Manifestations at very high doses include tonic pupils that are poorly responsive to light, suggesting significant paralysis of both autonomic pathways. Supersensitivity of the iris sphincter muscle to muscarinic agonists is a characteristic in severely intoxicated eyes, suggesting that antagonistic signaling via the sympathetic pathway is also impaired (Caya, 2001).

Unlike nerve agent, which stimulates the parasympathetic pathway, or BZ, which blocks the parasympathetic pathway, botulinum chemically denervates both signaling pathways at high doses, leading to poorly responsive or tonic pupils. In contrast, at lower systemic doses, BoNT preferentially blocks cholinergic neurotransmitter release, resulting in the dominance of the sympathetic signaling pathway.

The shellfish poison saxitoxin (STX) and the pufferfish poison tetrodotoxin (TTX) are also biological neurotoxins. Both toxins antagonize the voltage-gated sodium channels responsible for the rising phase of an action potential in neurons, thereby preventing action potential propagation and reducing or eliminating neurotransmitter release. Both toxins are exceedingly potent, with intravenous LD_{50} values of 10 and 8 μg/kg, respectively. The inhalational LD_{50} value for STX is estimated to be 5 $mg \cdot min/m^3$ (the equivalent value for TTX is not available). Although there are minimal data on toxic effects following topical exposure in healthy eyes, in principle the conjunctiva and corneal epithelium should provide an effective barrier to ocular absorption. Interestingly, topical administration of STX or TTX to deepithelialized corneas or by subconjunctival injection produces a long-lasting anesthetic effect, without causing ocular irritation or corneal edema (Duncan et al., 2001). In contrast, systemic administration can paralyze the ocular neurons, blocking pupillary light responses, spontaneous or reflexive eye openings, and gaze (Lan et al., 1999). At intermediate doses, patients may experience blurred vision, darkened vision, and loss of accommodation, with temporary blindness occurring at higher doses. There do not appear to be any chronic complications from ocular paralysis resulting from exposure to TTX or STX, and, upon recovery, oculomotor coordination, pupillary responses, and visual acuity were reported to be physiologically normal. These data suggest that the corneal epithelium and endothelium act as effective barriers to prevent the transcorneal absorption of TTX or STX, and therefore, ocular responses to aerosol exposures are more likely to result from inhalation and systemic distribution than from direct corneal absorption.

36.10.2 Ricin

Ricin is a heterodimer glycoprotein isolated from the castor bean. After nonspecific internalization into a wide variety of cells, ricin cleaves a glycosidic bond on the 60S subunit of eukaryotic ribosomes, preventing protein synthesis and causing cytotoxicity within 8 h. The median LD_{50} of ricin is around 22 μg/kg in humans from injection or inhalation. There is little information available on the ophthalmic manifestations of ocular exposure to ricin; however, topical administration to the eye is severely irritating, causing chemosis and conjunctivitis (Hunt, 1918; Grant and Schuman, 1993). Ocular instillation of 100 μg

to rabbits, guinea pigs, or mice causes extensive ocular inflammation that may lead to permanent corneal damage and blindness. It is unlikely that ricin can penetrate into the globe, and therefore the direct action of ricin must occur principally at the ocular surface (Strocchi et al., 2005).

36.10.3 *Staphylococcus* enterotoxin B (SEB)

SEB is a 23- to 29-kDa polypeptide in the bacterial superantigen family that cross-links the major histocompatibility class II receptor and T-cell receptors. This short-circuits the antigen processing and presenting mechanism, stimulating the release of pathologic levels of proinflammatory cytokines while failing to activate regulatory feedback signaling (Ahanotu et al., 2006). Prior to the 1975 Biological Warfare Convention (the biological equivalent of the CWC), SEB was studied by the United States as a biological agent that could be used to incapacitate soldiers in the battlefield. SEB was an attractive potential BWA because it can easily be aerosolized, is stable, and at low doses can cause widespread systemic damage when inhaled. In most circumstances, aerosol exposure results in a profoundly incapacitating illness lasting as long as 2 weeks, but it does not cause death (Sidell et al., 1997). SEB-induced ocular injuries are described only three times in the literature,; in all instances, exposures occurred when laboratory workers placed contaminated material in the near proximity to their eyes. Symptoms appear within 1−6 h, and included significant eyelid edema, periocular swelling, and acute conjunctivitis with discharge. Symptoms resolved within a week, although at least one patient appeared to exhibit continued hypersensitivity when in laboratories where SEB experiments were being conducted. These suggest that casualties resulting from an inhalational exposure may present with ocular toxicities that can be acutely incapacitating although, as with ricin, primarily localized to the ocular surface (Rusnak et al., 2004).

36.11 Concluding remarks and future directions

A wide array of CWAs and biological toxins exhibit acute ocular toxicities. The mode of action of these agents can be groupedinto three general categories: those that exhibit acute cytotoxic activity; those that stimulate symptomatic distress without directly causing cytotoxicity; and those that modulate neuro-ophthalmic signaling, thereby causing incapacitation, without directly injuring corneal tissues. The exquisite sensitivity of the eye to many of these agents enables the rapid appearance of ocular manifestations to serve as prodromic indicators of exposures. In the case of the CWAs that are most injurious to the eye—namely, vesicants—considerable research is still needed to identify therapeutic modalities that protect against long-term, progressive sequelae that can result in loss of vision. The ocular toxicities of the remaining agents are mild or reversible under most circumstances, and thus only temporarily incapacitating. However, one cannot discount the acute psychological distress of ocular pain or impaired vision, particularly if the causative agent is unknown and the prognosis is unclear. Thus, while most CWAs have transient and reversible effects on vision, preservation of vision remains a serious concern for a subset of potential agents.

Disclaimer

The views expressed in this chapter are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the US government.

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Chapter 37

Skeletal muscle

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37.1 Introduction

The skeletal muscles account for about half of a mammal's body weight, and, as a result, they receive proportionately large amounts of administered doses of chemicals. Skeletal muscle is a target organ for a variety of chemicals and toxic effects can range from minor muscle weakness or slight pain to complete paralysis. Skeletal muscles present major targets for the toxicity of organophosphate (OP) nerve agents, exceeded only by the brain. Morbidity and mortality associated with OP intoxication occur due to the effects of these compounds on skeletal muscles in general and the muscles of respiration in particular. Deaths from overdose of OPs are due in part to respiratory paralysis following depolarization of the neuromuscular blockade. Understanding the skeletal muscle system in the context of OP poisoning is essential, but it is also very complex because different muscle fiber types often respond differently, even to the same OP compound. The distinct features of slow and fast muscles are the most fascinating research aspects of skeletal muscles.

Both cholinergic and noncholinergic elements are present in the skeletal muscles, and are directly or indirectly modulated by OP nerve agents. Motor innervation plays an important role in the regulation of many properties of skeletal muscles, including neuromuscular activity. Changes in the activities of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) appear to greatly modulate neuromuscular activity and can modify neuromuscular transmission. At the cholinergic synapse, AChE plays an important role in the removal of acetylcholine (ACh) from the synaptic cleft. Inhibition of this enzyme by OP nerve agents profoundly modifies neuromuscular transmission, as seen in twitch potentiation, fasciculation, muscular weakness, and muscle cell death by necrosis or apoptosis. Because of their high metabolism, skeletal muscles are very vulnerable to OP-induced oxidative/nitrosative stress due to excess free radical generation. The importance of skeletal muscles has been recognized for decades because of their involvement in intermediate syndrome (IMS) and tolerance development related to the toxicity of OP pesticides. OP-induced effects on skeletal muscles can occur at one or multiple sites (the nerve fiber, the nerve terminal, the junctional cleft, the motor endplate, and the myofibrils). This chapter describes structural and functional aspects of skeletal muscles in the context of OP nerve agent toxicity.

37.2 Behavioral effects

Exposure to sublethal sign-producing doses of an OP nerve agent exerts prominent motor, behavioral, and autonomic symptoms. The motor symptoms are fasciculations, fibrillations, and body tremors. Fasciculations and fibrillations occur due to antidromic neural discharge from excess junctional ACh, while tremors are of a central origin (Gupta et al., 1986; Misulis et al., 1987). OP-induced behavioral studies indicate that different nerve agents [e.g., soman, sarin, tabun, and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX)] require different concentrations to produce equitoxic effects, degree of AChE inhibition, and myonecrosis. Acute symptoms of equal severity in male Sprague-Dawley rats by soman, sarin, tabun, and VX can be achieved at doses of 100, 110, 200, and 12 μg/kg, administered subcutaneously (s.c.), respectively (Gupta et al., 1987a,b, 1991). Based on the equitoxic doses, VX is the most toxic and tabun is the least toxic OP nerve agent.

With an acute dose of soman (100 μg/kg, s.c.) rats exhibited signs of toxicity, such as salivation, muscle fasciculations, and severe tremors, within 5−15 min.

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Signs of maximal toxicity appeared within 20−30 min, and persisted for about 4−6 h. Thereafter, the intensity is reduced to a mild form, but the signs of toxicity can still be observed after 24 h. Rats usually become free of overt signs after 72 h. With VX, onset of symptoms and appearance of maximal severity are delayed by approximately 20 min compared to other nerve agents (Gupta et al., 1987a,b, 1991). During peak toxicity of soman, signs such as complex posturing movements and tremors are indicative of pronounced CNS effects compared to moderate peripheral muscle fasciculations. Muscle fasciculations superimposed upon this activity are less prominent than the signs of gross motor unit activity. This is in contrast to the OP compound diisopropylphosphorofluoridate (DFP), which exerts greater peripheral activity with fasciculations and fewer central toxicity signs (Gupta et al., 1985, 1986, 1987a,b). This suggests that AChE inhibitors differ in their propensity to produce central or peripheral effects and that peripheral effects are required for muscle necrosis (Gupta et al., 1985, 1986, 1987a,b; Misulis et al., 1987).

37.3 Cholinergic system

Key elements of the cholinergic system include a neurotransmitter, ACh; an enzyme, AChE, which hydrolyzes ACh; and an enzyme, ChAT, which synthesizes ACh. All skeletal muscles contain these cholinergic components, but their quantities can significantly vary from muscle to muscle; that is, fast fiber-containing muscle has greater values than slow fiber- or mixed fiber-containing muscle.

37.3.1 Normal activity of acetylcholinesterase and its molecular forms

AChE (E.C. 3.1.1.7), in the muscle, is partly concentrated in the endplate region; that is, 20%−40% of the total amount found in the whole muscle (Hall, 1973; Younkin et al., 1982). Using a histochemical technique, Müntener and Zenker (1986) demonstrated the presence of AChE in the sarcoplasm of limited areas in sections of normal rat muscles. Normal activity of AChE varies from muscle to muscle; that is, higher AChE activity is found in fast fiber- (type II fibers in general, and type IIB fibers in particular) than in slow fiber-containing (type I fiber) muscle. Enzyme activity in mixed-fiber muscle (such as diaphragm) is found between the values of slow and fast muscles (Table 37.1). AChE plays an essential role in the removal of ACh at the neuromuscular junction (NMJ). Inhibition of AChE activity, which results in the accumulation of ACh, profoundly modifies neuromuscular transmission by producing fasciculations and twitch potentiation. At high rates of stimulation, the muscle is unable to maintain a normal contraction, and muscle hyperactivity often ensues in muscle fiber necrosis.

AChE exists in nerves and muscles in a range of globular and asymmetric molecular forms. A wide variety of sedimentation profiles have been established for AChE molecular forms in different mammalian muscles (Massoulie and Bon, 1982). The variations seen in the ratios of these molecular forms between different muscles are wide and complex. Qualitative and quantitative variations exist among different species, as well as the young versus adults (Barnard et al., 1984). In the rat extensor digitorum longus (EDL, a fast-twitch muscle), the G_1 (4S), G_4 (10S), and A_{12} (16S) molecular forms are predominant, while in the soleus (SOL, a slow-twitch muscle) and diaphragm (a mixed muscle), a fourth major molecular form is also present, called the A_8 (12S). In rat SOL, the majority of the total AChE activity is contributed by the 12S and 16S forms, whereas in the diaphragm, it is 4S and 10S, and in the EDL, it is 4S (Grosswald and Dettbarn, 1983a,b; Patterson et al., 1987) (Table 37.2).

In the rat, the 16S form is found in high concentrations at the endplates, and it is thought to be involved in neuromuscular transmission. The different molecular forms of AChE in SOL and EDL have apparent K_m values similar to that found in the diaphragm muscle (Grosswald and Dettbarn, 1983a,b). There appears to be no difference between catalytic sites of the molecular forms of AChE in fast-EDL and slow-SOL muscles, despite the different molecular form patterns and activity in these muscles.

TABLE 37.1 Normal values of AChE and BuChE activity in rat skeletal muscles.

Muscle	AChE activity (μmol substrate/g/h)	BuChE activity (μmol substrate/g/h)
EDL (fast fiber muscle)	105.8 ± 1.0	9.7 ± 0.8
Soleus (slow fiber muscle)	60.1 ± 1.2	10.3 ± 0.3
Diaphragm (mixed-fiber muscle)	78.3 ± 1.6	10.2 ± 0.2

The substrates used were acetylthiocholine iodide and butyrylthiocholine iodide for AChE and BuChE activity, respectively. Each value represents mean ± SEM ($n = 5–6$).

TABLE 37.2 Total AChE activity and percent contribution of molecular forms to total AChE activity in rat skeletal muscles.

			Molecular forms			
		Total activity	A_{12} (16S)	A_8 (12S)	G_4 (10S)	G_1 (4S)
EDL	w/ PI	93.8 ± 7.5	18.7 ± 1.8	–	26.4 ± 3.5	54.9 ± 4.1
	w/o PI	102.9 ± 4.7	18.7 ± 1.6	–	30.2 ± 2.8	51.1 ± 3.1
Soleus	w/ PI	53.6 ± 4.7	26.8 ± 1.9	33.7 ± 2.3	15.2 ± 1.0	24.3 ± 0.9
	w/o PI	61.4 ± 5.3	25.9 ± 2.2	33.0 ± 2.2	16.3 ± 1.3	24.8 ± 1.4
Diaphragm	w/ PI	81.4 ± 6.1	26.9 ± 1.4	14.2 ± 0.9	28.8 ± 0.3	30.1 ± 1.7
	w/o PI	89.5 ± 4.9	24.6 ± 1.8	14.8 ± 0.9	29.7 ± 1.7	30.9 ± 1.5

Activity values are expressed as μmol ACh hydrolyzed/g/h tissue for total activity and as a percentage of the total distribution for molecular forms. Values are the mean ± SD ($n = 6–8$ muscles); w/ PI, protease inhibitors present; w/o PI, protease inhibitors absent. No statistical significance was found comparing the presence versus absence of protease inhibitors for values of the individual molecular forms or total activities.

37.3.2 Inhibition of acetylcholinesterase and its molecular forms by nerve agents

Following an acute exposure to OP nerve agents, signs of cholinergic toxicity may appear within a few minutes and are caused by irreversible inhibition of AChE activity in neuronal tissues. Inhibition of AChE causes excess accumulation of ACh at central and peripheral synaptic sites, leading to failure of neuromuscular, respiratory, and cardiovascular functions. For some OP compounds, a close relationship exists between the severity of toxic signs and the inhibition of AChE during the acute phase of intoxication. The observable toxic effects usually do not persist for more than 4–6 h, while recovery of AChE activity occurs at a much slower rate, such as 7–14 days or even 3–4 weeks, depending on the tissue and inhibitor.

Gupta et al. (1987a) demonstrated that selective inhibition of AChE activity in skeletal muscles was apparent within 1 h of soman administration (100 μg/kg, s.c.) in rats when SOL showed the maximum inhibition (87%), whereas EDL showed the least inhibition (47%). AChE activity in skeletal muscles was maximally depressed during the following 3–6 h, the time when animals showed severe signs of toxicity. Recovery was clearly apparent during the next 3–7 days in all three skeletal muscles (SOL, EDL, and diaphragm); 7 days after soman treatment, enzyme recovery was greater than 90% in EDL and diaphragm compared to 75% in SOL. Within 1 h, all molecular forms of AChE were reduced to less than 10% of control in SOL and diaphragm (Fig. 37.1). In EDL, the 16S form, mainly localized at the NMJ, was not affected at this time, while the 10S form was completely inhibited and the 4S form was reduced to 50%. Further inhibition was seen after 24 h but even then the 16S form was the least inhibited in EDL. After 3 days of soman treatment, the AChE molecular forms in all three muscles showed signs of recovery. This was particularly evident in the 16S form in EDL and diaphragm, where it appeared to be approximately 75% of controls. In the SOL, the 16S form had recovered to only about one-third of the control when measured after 3 days; 7 days after soman, the 16 and 4S forms in the EDL and diaphragm had fully recovered. The activities of the 10S EDL form and the 10 and 12S diaphragm forms were still reduced, while in the SOL the activity of all forms remained below the control.

In similar experiments, tabun (200 μg/kg, s.c.) produced differential AChE inhibition in various skeletal muscles (SOL = diaphragm > EDL), which was similar to that seen with soman (Gupta et al., 1987a,b). The varying degrees of AChE inhibition in the skeletal muscles treated with tabun, however, correlated well with the observed difference in the number of myonecrotic lesions; that is, the greater the AChE inhibition (during the initial 24-h period), the higher the number of lesions found in the diaphragm and SOL. EDL, with a low level of AChE inactivation, had the smallest number of lesions. This observation was in agreement with a similar pattern of histochemical observations following soman and sarin administration (Meshul et al., 1985; Gupta et al., 1987a, 1991). The reason for this difference in susceptibility to a particular nerve agent may be due to: (1) variations in the location of AChE in different muscles (Grosswald and Dettbarn, 1983a), (2) changes in ACh release due to a different firing pattern (Misulis et al., 1987), and (3) pharmacokinetic variables, which influence the delivery of a particular OP compound. With tabun toxicity, inhibition and recovery of AChE molecular forms, especially the 16S form associated with the NMJ (endplate region),

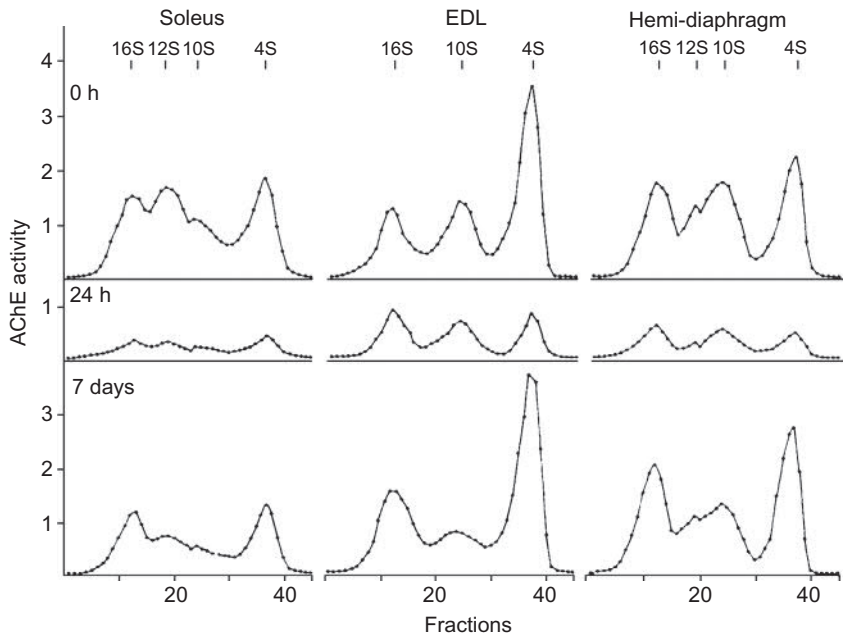

FIGURE 37.1 Representative profiles of the activity of the AChE molecular forms in the SOL, EDL, and hemidiaphragm muscles. Profiles at the top of each column are from untreated muscles, followed by profiles of activity of AChE molecular forms of muscles 24 h and 7 days after receiving an acute dose of soman (100 μg/kg, s.c.). The AChE activity scale is in arbitrary units based on the micromole substrate hydrolyzed/min by the enzyme activity in each fraction. The sedimentation values of the AChE molecular forms are given in the profiles of untreated muscles above the associated peaks. Sedimentation values were determined by the location of the added sedimentation standards, β-galactosidase (16.0S), catalase (11.1S), and alkaline phosphatase (6.1S), following velocity sedimentation of the gradients.

corresponded well with the appearance and disappearance of necrotic lesions. The delay in the molecular form's return to normal is due to the fact that the heavier molecular forms (16, 12, and 10S) are based on an assembly of the monomeric 4S form. Synthesis of the 4S form increases when the assembly of the heavier forms lags behind.

Following an acute exposure to sarin (110 μg/kg, s.c.), AChE activity in skeletal muscles was reduced to 23% in SOL and 48% in diaphragm within 1 h, while EDL AChE was significantly unaffected. By 24 h, however, a still-greater inhibition was seen for these muscles, and in EDL, AChE activity was reduced to 43%. In an early phase, recovery of AChE was rapid, but still not complete when measured after 7 days of treatment. Activities of the AChE molecular forms, after 1 h of sarin injection, were significantly depressed in the SOL and diaphragm, while those in the EDL showed significant inhibition only after 24 h (Fig. 37.2). By day 7 in SOL, activities of the 4 and 10S molecular forms of AChE had recovered to higher than control levels, while in the diaphragm, a significant shift toward the 4S molecular form had taken place so that the AChE profile resembled that of a control EDL. In EDL, the activity of 4 and 16S molecular forms recovered at a faster rate than the 10S molecular form.

Following VX administration (12 μg/kg, s.c.), within 1 h, AChE activity of all three skeletal muscles was reduced to between 8% and 17% of control with incomplete recovery by the end of 7 days. At the same time, VX caused significant inhibition in the activity of all molecular forms in all three muscles (Fig. 37.3). In EDL, the activity of 4 and 10S showed significant recovery 24 h later, while those of SOL and diaphragm remained inhibited. By day 7, the activity of all forms had recovered and an excess of activity was seen in the 4S form of EDL and diaphragm, shifting the latter profile toward that of the EDL and contributing a higher protection to the total AChE activity.

37.3.3 Butyrylcholinesterase

Butyrylcholinesterase (BuChE, E.C. 3.1.1.8) activity in skeletal muscles is significantly lower than AChE activity (Table 37.1). Also, unlike variable AChE activity, all three muscles (SOL, EDL, and diaphragm) contain equal levels of BuChE activity. Following soman administration (100 μg/kg, s.c.) in rats, maximal inhibition of BuChE activity in skeletal muscles was observed after 24 h. At this time, SOL was greatly affected (98%), followed by the diaphragm (87%) and EDL (60%). A rapid recovery of BuChE was noticed during 48−72 h after soman treatment and enzyme activity returned to baseline values when measured after day 7 (Gupta et al., 1987a).

Tabun (200 μg/kg, s.c.) caused significant inhibition of BuChE activity within 1 h in skeletal muscles (SOL, EDL, and diaphragm, 14%, 50%, and 35% remaining activity, respectively), but maximal inhibition appeared after 3 h in SOL (6% remaining activity), and after 24 h in diaphragm and EDL (7% and 11% remaining activity, respectively) (Gupta et al., 1987b). Unlike AChE, the recovery rate of BuChE appeared to be rapid, as the enzyme activity in the diaphragm and EDL recovered to baseline values by day 7. At this time, SOL BuChE activity remained significantly inhibited. After 1 h of sarin

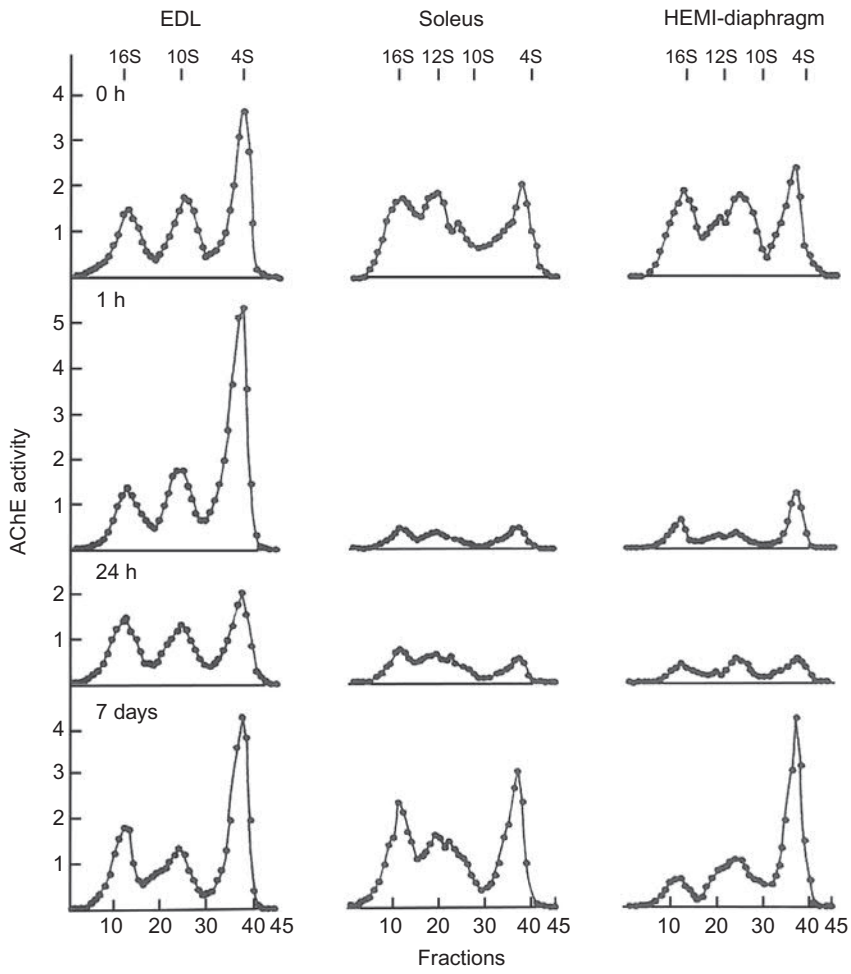

FIGURE 37.2 Representative profiles of the AChE molecular forms in the EDL, SOL, and hemidiaphragm muscles from rats following an acute sublethal injection of sarin (110 μg/kg, s.c.). Profiles at the top of each column are from untreated muscles, and subsequent profiles show the activity of the AChE molecular forms 1 h, 24 h, and 7 days, respectively, after sarin treatment. For further details, see Fig. 37.1.

administration (110 μg/kg, s.c.), BuChE activity was significantly inhibited in the SOL and EDL (78% and 79% remaining activity, respectively), but not in the diaphragm. With sarin, maximal BuChE inhibition was noted after 24 h in SOL, EDL, and diaphragm (39%, 71%, and 43% remaining activity, respectively). In similar studies, within 1 h VX (12 μg/kg, s.c.) caused significant and maximal inhibition of BuChE in all three muscles (29%, 60%, and 60% remaining activity, respectively). When measured after 24 h, EDL and diaphragm showed marked recovery, while SOL still had 41% inhibition. All three muscles showed complete recovery of BuChE when measured after 7 days.

37.3.4 Choline acetyltransferase

Existence of ChAT (acetyl-CoA-choline *O*-acetyltransferase, EC 2.3.1.6) in skeletal muscles is probably of neural origin, and its activity varies among skeletal muscles. ChAT activity can be altered by increased or decreased neuromuscular activity. It appears that neuromuscular activity exerts a regulatory influence on neuronal production of ChAT. Alterations in ChAT activity in response to variations in muscular activity represent changes in enzyme synthesis, although the effects on catabolism of the enzyme or on exoplasmic transport of enzyme to the nerve terminal cannot be ruled out. Acute exposure to DFP (1.5 mg/kg, s.c.) caused insignificant increase, while repeated administration at a low dosage (0.5 mg/kg/day for 5 days) resulted in significant increase in ChAT activity (diaphragm 140%, EDL 150%, and SOL 156%). A significant increase in ChAT activity was noted even after 2 weeks of repeated administration.

37.3.5 Acetylcholine receptors

Acetylcholine receptors (AChRs) are of two types: muscarinic (mAChR) and nicotinic (nAChR), based on the agonist activities of the natural alkaloids, muscarine and nicotine, respectively. These receptors are functionally different. The muscarinic type being G-protein-coupled receptors mediate a slow metabolic response via second

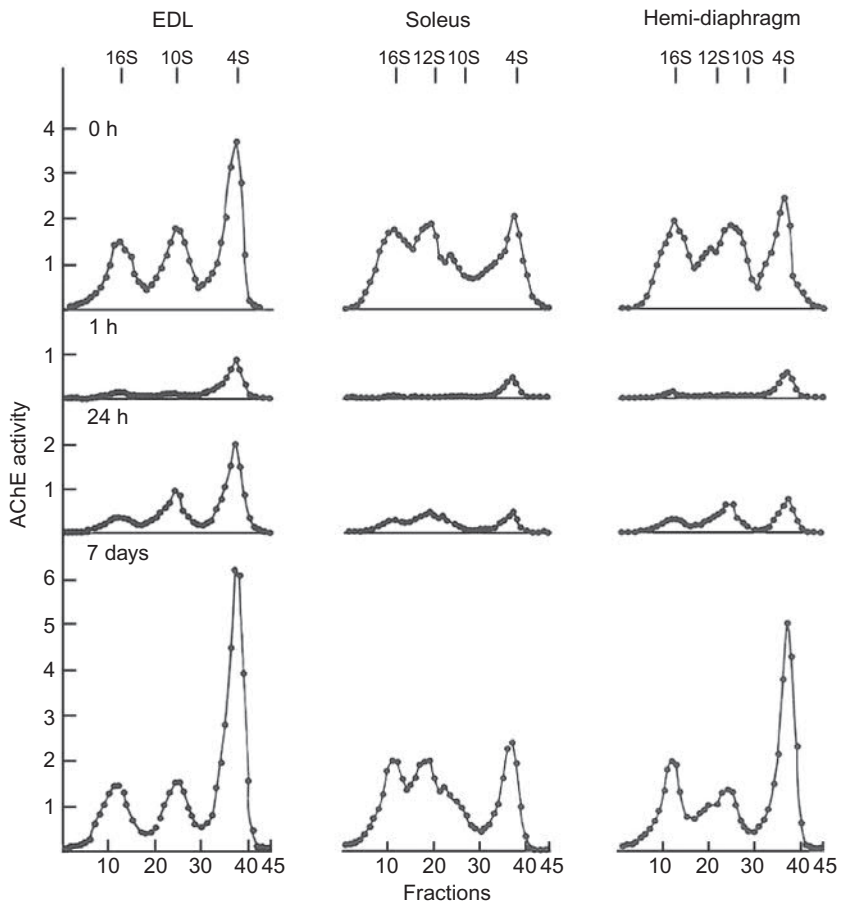

FIGURE 37.3 Representative profiles of the activity of the AChE molecular forms in the EDL, SOL, and hemidiaphragm muscles from rats following an acute, sublethal injection of VX (12 μg/kg, s.c.). For further details, see Fig. 37.1.

messenger cascades, while the nicotinic type are ligand-gated ion channels, which mediate a fast synaptic transmission of the neurotransmitter. Skeletal muscles are enriched with nAChRs and are devoid of mAChRs.

The nAChRs, with a molecular mass of 290 kDa, are composed of five receptor subunits (α, β, δ, ε, and γ) arranged symmetrically around the central pore. Adult muscle is composed of α2βαδγ, whereas fetal muscle has α2βδε (Mishina et al., 1986). Experimental studies in the late 1970s revealed that in relatively immature muscle cells, the AChRs are distributed uniformly over the entire sarcolemma (Bevan and Steinbach, 1977). At about the time of the initial innervation, aggregations/clusters of AChRs appear, in addition to the relatively low-density receptors (Braithwaite and Harris, 1979). During the course of embryonic development, the low-density receptors disappear, and almost all AChRs in the adult muscle fibers are localized to the region of the NMJ (Fambrough, 1979). Sohal and Boydston (1982) suggested that neither the total receptor content nor the ability of the receptors to cluster seems an essential element for the formation and maintenance of the morphological aspects of the NMJs or for the growth and maturation of the muscle. It is yet to be established what exactly, if any, role nAChRs have in the formation of the NMJ.

At the mammalian NMJ, where nAChRs are localized, stimulation of the presynaptic nerve causes release of the neurotransmitter ACh from the nerve terminals. Released ACh diffuses across the gap, separating nerve and muscle cells and interacts with specific receptors associated with the postsynaptic muscle membrane to produce increased membrane permeability to some cations. When two molecules of ACh bind to an nAChR, a conformational change occurs in the receptor, resulting in the formation of an ion pore. The nAChRs are a family of cationic channels whose opening is controlled by ACh and nAChR agonists and they are the key molecules in cholinergic nicotinic transmission at the NMJ. At this site, the opening of a pore produces a rapid increase in the cellular permeability of Na^+ and K^+ ions, resulting in the depolarization and excitation of the muscle cell, thereby producing a muscular contraction. Adult vertebrate muscle fibers are highly

sensitive to ACh only in the region of the NMJ. Muscle fibers of fetal and neonatal rats are also sensitive to ACh in regions outside the NMJ.

Excessive ACh or long-acting cholinomimetic agents can produce muscle paralysis due to prolonged depolarization of the endplate, a phenomenon referred to as depolarization block. Prolonged transmitter (ACh) receptor (nAChR) interactions, as a result of the high concentrations of accumulated ACh, produce a depolarization block, similar to that seen with agents such as decamethonium. Furthermore, there is a possibility of the same postjunctional area being activated repeatedly, resulting in desensitization of receptors (desensitization block). These forms of neuromuscular block usually produce muscle weakness and paralysis.

The binding site for cholinergic effectors is believed to reside primarily in the α-subunits. Evidence for this fact is based on affinity-labeling experiments in which analogs of ACh [bromoacetylcholine and 4-(-*N*-maleimidophenyl) trimethylammonium] have been shown to label only the α-subunits. Receptors also demonstrate high-affinity binding of the polypeptide α-neurotoxins, such as α-bungarotoxin (αBT), which interacts specifically and in an essentially irreversible manner with nAChRs. At the nAChRs, αBT competes for binding with both cholinergic agonists (e.g., carbamylcholine chloride and decamethonium bromide) and antagonists (e.g., curare) Berg et al. (1972) reported binding of αBT to AChRs in rat diaphragm muscle and found that 90% of the binding that occurred was "endplate-specific."

Some of the toxic effects of OP nerve agents and pesticides are unrelated to inhibition of AChE. However, the exact mechanism by which OP nerve agents and other anti-AChE compounds affect nAChRs is yet to be elucidated. Some of the biochemical and morphological alterations have been attributed to an excess amount of ACh in the synaptic cleft resulting from AChE inactivation. Many anti-AChE agents have been shown to produce postsynaptic morphological, biochemical, and electrophysiological alterations in adult mammalian skeletal muscle after acute and long-term treatment (Fenichel et al., 1972; Laskowski et al., 1977; Hudson et al., 1978). Normally, at the motor endplate, the small electrical nerve impulse releases ACh, which diffuses across the synaptic membrane and attaches to the postsynaptic receptors on the muscle membrane. However, during AChE inhibition by OPs, unhydrolyzed ACh does not diffuse from the cleft, but repeatedly combines with postsynaptic receptors. The prolonged presence of ACh in the synaptic area appears to cause some of the myopathic changes. This was supported by observations of paraoxon, causing antidromic firing and increasing spontaneous miniature endplate potential (MEPP) frequency to 38 times the control rates (Laskowski and Dettbarn, 1971).

Gupta et al. (1986) demonstrated that an acute exposure to DFP (1.5 mg/kg, s.c.) changed neither nAChR density (B_{max}) nor the affinity constant (K_D) in the diaphragm muscle. Subchronic DFP treatment (0.5 mg/kg/day, s.c., for 5 days) caused a marked decrease in B_{max} (56%), without a significant change in K_D. Chronic treatment with anti-AChEs reduced the total number of nAChRs by 42%−45% in the endplate region (Chang et al., 1973). Later studies revealed that in sublethal doses, OPs induce symptoms that cannot be solely attributed to AChE inhibition, indicating a direct interaction with postsynaptic nAChRs (Menking et al., 1990). Tattersall (1990) investigated the effects of DFP, sarin, soman, VX, and echothiophate on the nAChR ion channel at the adult mouse muscle endplate by using single-channel recording techniques. DFP, sarin, and soman had no effect on open times at concentrations up to 100 μM, but echothiophate and VX were found to have voltage- and concentration-dependent, open-channel blocking at concentrations of 1−50 μM. In similar experiments, Bakry et al. (1988) demonstrated that OP nerve agents and echothiophate bind to AChRs, inhibit or modulate binding of radioactive ligands to these receptors, and modify events regulated by them. The OPs also bound to allosteric sites on the nAChR (identified by inhibition of ^{3}H-phencyclidine bonding), but some bound also to the receptor's recognition site (identified by inhibition of ^{125}I-αBT binding). Soman and echothiophate in micromolar concentrations acted as partial agonists of the nAChR and induced receptor desensitization. On the other hand, VX acted as an open-channel blocker of the activated receptor and also enhanced receptor desensitization. Membrane fragments from torpedo electric organs were used to determine these interactions using ^{3}H-phencyclidine as a probe. The results were consistent with the hypothesis that OPs bind to, and irreversibly phosphorylate, an allosteric site on the ion channel associated with the nAChR.

In the context of OP toxicities, it needs to be emphasized that for a normal muscle contraction, the ratio of AChE to nAChRs is crucial in determining the minimum AChR density. Since the AChE recovers at almost the same rate as do the AChRs, a balance of AChE to AChR is maintained over the postsynaptic surface during recovery. A relatively constant ratio of AChE to AChR is very important for maintaining normal neuromuscular function.

37.4 Noncholinergic system

37.4.1 Muscle excitotoxicity

Involvement of the cholinergic neurotransmitter ACh in muscle excitotoxicity has been known for a long time.

There is also mounting evidence showing the presence of glutamatergic machinery (receptors, transporters, and glutamate itself) at the NMJ (Pinard and Robitaille, 2008). It has been suggested that glutamate and ACh are coreleased at the NMJ. Glutamate might be a mediator or modulator of neuromuscular transmission. Glutamate receptors present at the NMJ are predominantly an *N*-methyl-D-aspartate (NMDA) subtype. Furthermore, it was demonstrated in in vivo studies that a noncompetitive NMDA receptor (NMDAR) antagonist, known as memantine, blocks muscle fasciculations induced by AChE inhibitors (DFP, soman, sarin, tabun, and VX), suggesting an involvement of NMDARs (Gupta and Dettbarn, 1992; McLean et al., 1992). Of course, memantine exerts several additional pharmacological actions, including nAChR blockage. Unlike the well-understood role of cholinergic excitotoxicity, the role of glutamate excitotoxicity is yet to be established in AChE inhibitor-induced muscle toxicity.

37.4.2 Oxidative/nitrosative stress

Although the exact mechanism underlying skeletal muscle damage by OP nerve agent-induced hyperactivity still remains unclear, evidence clearly indicates that free radicals play an important role. During normal conditions, free radicals are generated at a low rate and subsequently taken care of by the well-developed scavenger and antioxidant systems. However, during exhaustive hyperactivity of the skeletal muscles caused by anti-ChE, excessive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (hereafter referred to collectively as ROS) are generated and exceed the capacity of the muscle defense system, thus producing oxidative stress. Excessively generated ROS can cause muscle injury by reacting with cellular components, such as membrane phospholipids, mitochondrial enzymes/proteins, and nucleic acids. Consequently, this leads to skeletal muscle cell apoptosis/necrosis, inflammation, and loss of cell viability.

A causal relationship appears to exist between excitotoxicity, ROS, lipid peroxidation, and muscle cell injury/death. One of the well-recognized targets of ROS-induced injury is peroxidation of lipids and the formation of prostaglandin F_2-like compounds, such as F_2-isoprostanes (F_2-IsoPs) (Milatovic et al., 2019). Studies have shown that the assay of these compounds provides an accurate measure of lipid peroxidation (Roberts and Morrow, 2000; Dettbarn et al., 2001). Quantification of F_2-IsoPs and nitric oxide (NO) in skeletal muscles (SOL, EDL, and diaphragm) has provided strong evidence that AChE inhibitor-induced toxicity initiates lipid peroxidation and muscle cell injury (Dettbarn et al., 2001; Gupta et al., 2001a,b; Milatovic et al., 2001). Yang and Dettbarn (1998) found a significant increase of F_2-IsoPs in diaphragm muscle (156%) 1 h after DFP injection (1.7 mg/kg, s.c.), when muscle hyperactivity was maximal.

The primary reason for the increased generation of ROS appears to be a decreased rate of ATP synthesis in the mitochondria, which is related to a loss of cytochrome oxidase (COX) activity. COX is the terminal complex in the mitochondrial respiratory chain, which generates ATP by oxidative phosphorylation. During intense muscle hyperactivity, the activity of COX is reduced, leading to an increase in the electron pressure within the electron transport chain and to increased ROS production (Yang and Dettbarn, 1998). More than 90% of O_2 consumption in the cells is catalyzed by COX. The chance of intermediary products, such as superoxide anion, hydrogen peroxide, and the hydroxyl radical, escaping is small under conditions where COX remains active. A reduced capacity of this enzyme, however, increases the risk for an incomplete reduction of O_2 and further O_2 radical formation.

Xanthine oxidase (XO) is another enzyme that contributes to increased ROS generation. During normal conditions, 80%–90% of native XO exists as xanthine dehydrogenase (XD), but during metabolic stress and increased Ca^{2+}, XD is converted to a reversible oxidase form. XO uses molecular O_2 instead of NAD^+ as an electron acceptor. Molecular O_2 is thereby reduced and the superoxide radical (O_2^-) is formed. During hyperactivity of the muscle by AChE inhibitors, regeneration of ATP is insufficient, not due to the lack of O_2, but due to greater utilization and impaired synthesis of ATP (Gupta et al., 1994, 2000a,b, 2001a, 2002). Unlike ischemia, O_2 is present during oxidative stress caused by prolonged contractile activity. This suggests that subsequent to the conversion of XD to XO, O_2 is continuously univalently reduced to superoxide anions. This occurs during oxidative stress when ATP utilization exceeds the rate of ATP synthesis during increased muscle activity. Yang and Dettbarn (1998) provided direct evidence for the role of COX and XO in muscle injury by its hyperactivity, showing that during DFP-induced muscle hyperactivity, a decrease in COX activity and an increase in XO activity occurred. The NAD(P)H oxidases can also be the major O_2^--generating sources in contracting skeletal muscle (Sakellariou et al., 2014). Blockage of muscle fasciculations prevented these enzyme changes.

Another ROS contributing to oxidative stress is peroxynitrite ($ONOO^-$), which is formed by the reaction of NO with superoxide (O_2^-). $ONOO^-$ has the potential to modify biomolecules through several different mechanisms and is a good candidate for mediation of the NO-dependent pathophysiological process. Under normal conditions, NO is widely regarded as a multifunctional messenger/signaling molecule and is thought to have two

physiological functions in skeletal muscle. The first is to promote relaxation through the cGMP pathway, and the second is to modulate muscle contractility that depends on reactive oxygen intermediates. At the NMJ, NO appears to be a mediator of: (1) early synaptic protein clustering, (2) synaptic receptor activity and transmitter release, and (3) downstream signaling for transcriptional control (Blottner and Luck, 2001). NO has also been demonstrated to modulate excitation−contraction coupling in the diaphragm muscle (Reid et al., 1998). Within skeletal muscle cells, all three known nitric oxide synthase (NOS) isoforms (neuronal, nNOS; endothelial, eNOS; and inducible, iNOS) are present, but nNOS, which is Ca^{2+} dependent, seems to predominate, and is concentrated at the sarcolemma and postsynaptic surface of the NMJ (Stamler and Meissner, 2001). In a recent study, NOS has been demonstrated to play the role of mechanosensor in skeletal muscle fibers (Smith et al., 2002). Neuronal NOS in skeletal muscles is involved in the regulation of metabolism and muscle contractility. Kobzik et al. (1994) reported for the first time that the NOS activity of individual muscles could be correlated primarily with type II fibers (being highest in the EDL), while Frandsen et al. (1996) found the distribution of nNOS homogeneous.

Data presented in Table 37.3 show the levels of citrulline (determinant of NO/NOS) in skeletal muscles of control rats and those treated with an acute dose of DFP (1.5 mg/kg, s.c.). In control muscles, markedly higher citrulline levels were found in the SOL, followed by the EDL and diaphragm. The observed higher level of NO in the SOL could be due to a greater activity of NOS. Within 1 h of DFP exposure, when rats exhibited signs of peak severity and maximal reduction of AChE activity (90%−96%), the levels of citrulline were maximally increased (272%−288%), and remained significantly elevated in all three muscles (>twofold) when measured after 2 h. The finding of elevated NO by DFP-induced muscle hyperactivity (Gupta et al., 2002) was supported by previous studies showing that increased muscle contractility generates significantly greater quantities of ROS/RNS (Yang and Dettbarn, 1996; Clanton et al., 1999). A significant increase in NO is known to cause the inhibition of mitochondrial function and thereby appears to be the cause of the impaired synthesis of ATP.

37.4.3 High-energy phosphate depletion and myonecrosis

The interest in the role of energy metabolites (especially ATP) in chemical-induced mitochondrial cytotoxicity has been reinvigorated, since ATP appears to be a switch to decide whether cells die from apoptosis or necrosis. Muscle necrosis is probably caused by increased contractile activity in individual muscle fibers. Ca^{2+} is believed to regulate mitochondrial oxidative phosphorylation, thereby contributing to the maintenance of cellular energy homeostasis (Glancy et al., 2013). In normal cells, the mitochondrial Ca^{2+} content is relatively low compared to that in the cytoplasm. However, abnormally high cytosolic Ca^{2+} levels (due to NMDAR activation and impaired Ca^{2+} extrusion, which is an energy-dependent process) perturb many cellular processes. The major changes include: (1) reduced COX activity, (2) increased XO activity, (3) mitochondrial damage due to excessive mitochondrial Ca^{2+} accumulation, (4) reduced ATP synthesis, and (5) increased production of O_2^- and NO^-, resulting in increased $OONO^-$ formation. The extreme vesiculation and disruption of the sarcoplasmic reticulum (SR) under the endplate, as well as the swollen mitochondria may reflect Ca^{2+} overloading of the muscle Ca^{2+}-binding capacity, which could result in high sarcoplasmic Ca^{2+} levels, which in turn leads to necrosis (Salpeter et al.,1982). The control of intracellular Ca^{2+} concentration is of great importance to muscle fibers, and a transient rise leads to contraction. An increased net influence of Ca^{2+} forces the mitochondria and SR to maintain Ca^{2+} homeostasis by sequestering the excessive amounts of this ion. This is an energy-consuming process and occurs in preference to ATP formation. The ensuing lack of energy ultimately causes the free sarcoplasmic Ca^{2+} to rise and

TABLE 37.3 Citrulline levels (nmol/g) in skeletal muscles of rats after 1 h of DFP (1.5 mg/kg, s.c.) injection.

Treatment	Soleus	EDL	Diaphragm
Control	451.2 ± 5.3	381.3 ± 8.2	331.2 ± 9.9
	(100%)	(100%)	(100%)
DFP	1227.1 ± 47.2[a]	1061.0 ± 38.9[a]	952.3 ± 49.8[a]
	(272%)	(278%)	(288%)

Values are means ± SEM ($n = 4-5$).
[a]*Significant difference between control and DFP-treated rats* ($P < .05$).

TABLE 37.4 Energy phosphates and their metabolites in muscles of rats intoxicated with DFP (1.5 mg/kg, s.c.).

	Treatment	μmol/g (means ± SEM; $n = 4–5$)			
		ATP	TAN	PCr	TCC
Soleus	Control	3.42 ± 0.05	4.42 ± 0.04	6.38 ± 0.19	20.41 ± 0.37
		(100)	(100)	(100)	(100)
	DFP	2.06 ± 0.06[a]	2.78 ± 0.08[a]	3.67 ± 0.19[a]	12.87 ± 1.01[a]
		(60)	(63)	(57)	(63)
EDL	Control	4.91 ± 0.37[b]	5.80 ± 0.38[b]	9.63 ± 0.59[b]	26.30 ± 1.64[b]
		(100)	(100)	(100)	(100)
	DFP	3.42 ± 0.02[a]	4.15 ± 0.02[a]	6.82 ± 0.23[a]	21.74 ± 0.33[a]
		(70)	(72)	(71)	(83)
Diaphragm	Control	4.47 ± 0.23	5.76 ± 0.26	6.18 ± 0.03	23.34 ± 0.17
		(100)	(100)	(100)	(100)
	DFP	2.74 ± 0.43[a]	3.66 ± 0.05[a]	3.96 ± 0.12[a]	15.86 ± 0.45[a]
		(61)	(64)	(64)	(68)

[a]*Significant difference between control rats and DFP-treated rats ($P < .05$).*
[b]*Significant difference between soleus and EDL values of control rats ($P < .05$); numbers in parentheses are percent remaining values compared to controls (100%).*

an excessive ROS formation, leading to necrosis. The energy required for muscle contraction is derived from the breakdown of ATP. As soon as ATP is broken down, it is promptly restored in the so-called Lohmann reaction at the expense of phosphocreatine (PCr).

Data of high-energy phosphates (i.e., ATP and PCr) and their metabolites in the skeletal muscle of control rats and those treated with an acute dose of DFP (1.5 mg/kg, s.c.) are shown in Table 37.4. Analyses of control muscles revealed the levels of ATP and PCr to be higher in the EDL, followed by the diaphragm and the SOL. The values of energy charge potential (ECP = ATP + 0.5ADP/TAN) in SOL, EDL, and diaphragm were 0.86 ± < 0.01, 0.91 ± < 0.01, and 0.86 ± < 0.01, respectively. At the time of maximal severity (i.e., 1 h after DFP exposure), the levels of ATP, total adenine nucleotides (TAN = ATP + ADP + AMP), PCr, and total creatine compounds (TCC = PCr + Cr) were maximally reduced in all three muscles, and they remained reduced to the same degree after 2 h. Similar results were found with soman toxicity.

Depletion of energy-rich phosphates (ATP and PCr) by AChE inhibitors occurs due to impaired synthesis and greater utilization of ATP during muscle hyperactivity. In fact, the time course of necrosis correlates with the reduced levels of PCr, the reduction of which may have been the result of an increased demand for energy and a low rate of ADP phosphorylation, caused by an increased level of sarcoplasmic Ca^{2+}. AChE inhibitor-induced increases in NO (Table 37.3) can exert cellular toxicity primarily by depleting energy stores through multiple mechanisms: (1) by prolonging poly-(ADP-ribose) polymerase activation; (2) by inhibiting mitochondrial enzymes, such as COX, aconitase, and creatine kinase (CK); and (3) by inhibiting the glycolytic enzyme phosphofructokinase. NO, at nanomolar concentrations, can directly and specifically inhibit mitochondrial respiration by competing with molecular O_2 for binding to COX, thereby causing an inhibition of ATP synthesis (Brown and Cooper, 1994). Other factors that contribute to the decline of energy metabolites may include damage to mitochondria, a higher rate of ATP utilization needed to generate NAD^+ in the ADP-ribosylation of nuclear proteins, enhanced influx of sarcoplasmic Ca^{2+}, an increased number of contractile protein cross-bridges, and the release of ATP in concert with ACh from the nerve terminals. The net effect of AChE inhibitor-induced muscle hyperactivity is a reduced cellular energy level.

37.5 Muscle activity—electromyography

AChE inhibitors (DFP, 1.5 mg/kg, s.c.; or soman, 100 μg/kg, s.c.) at a toxic sign-producing sublethal dose elicit prominent motor, behavioral, and autonomic symptoms. As mentioned earlier, the motor symptoms are fasciculations, fibrillations, and body tremors. Fasciculations and fibrillations are due to

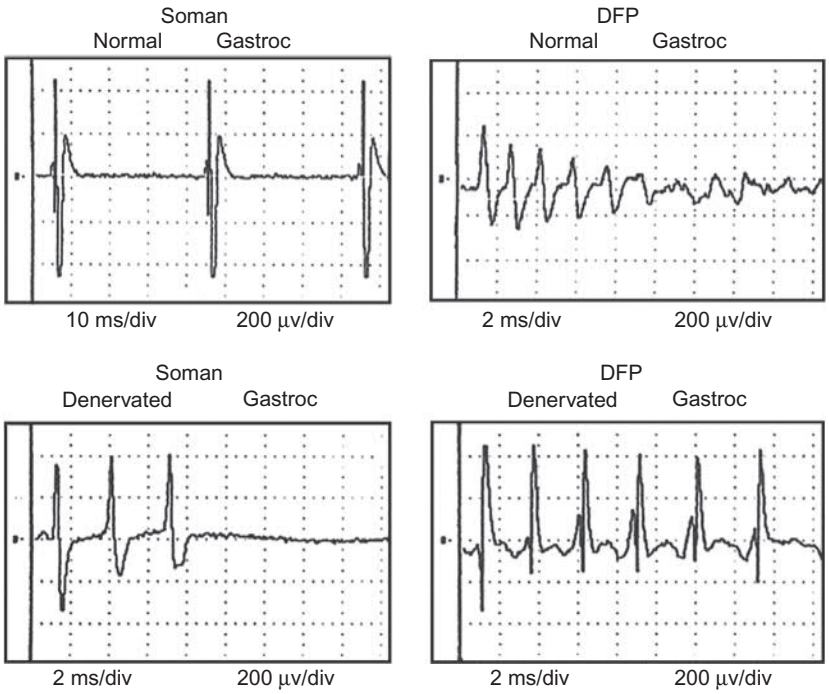

FIGURE 37.4 EMG recordings from normal and acutely denervated lateral gastrocnemius in response to soman (100 µg/kg, s.c.) or DFP (1.5 mg/kg, s.c.). Left, soman; right, DFP; top, non-denervated muscle; bottom, denervated muscle. Note the difference in time base and voltage scale. The differing morphology of the normal DFP waveform occurs due to electrode orientation and distance from the muscle fiber and does not represent a systematic difference between the effects of individual agents.

antidromic neural discharge from excess junctional ACh, while tremors are of a central origin. Misulis et al. (1987) demonstrated a difference in the pattern of motor symptoms in rats treated with soman or DFP. Soman produces complex posturing movements and tremors affecting virtually the entire body, while DFP produces movements that are similar to fasciculations or myokymia. Muscle fiber necrosis is also more frequent at symptom-producing doses of DFP than equivalent doses of soman. This suggests that the AChE inhibitors differ in their propensity to produce central or peripheral effects and that peripheral effects are required for muscle necrosis.

Electromyographic (EMG) findings indicate that soman and DFP produce different responses (Fig. 37.4). The majority of the motor symptoms induced by soman are due to impulses descending from the CNS, and a proportion of these symptoms are epileptiform activity. This activity is not generated at spinal levels. In contrast, DFP produces motor symptoms mainly by peripheral action that depends on a functioning nerve terminal (Misulis et al., 1987). Anderson (1987) also demonstrated that DFP and soman have opposite effects on skeletal muscle contracture during tetanic stimulation. DFP significantly decreased the ability of rat skeletal muscle to maintain a contracture. Soman, on the other hand, increased muscle force in a frequency-dependent manner. The opposite actions of DFP and soman on muscle contractility might be the consequence of a difference in effects of these two agents on postsynaptic muscle ACh receptors. The finding of Anderson (1987), which indicated an increase in contracture following soman, was somewhat surprising in view of the work of Dettbarn (1984) and Gupta et al. (1987a,b), who reported that soman is as effective as other AChE inhibitors in producing muscle necrosis (Table 37.6). However, Meshul et al. (1985) reported that soman appears to spare motor endplates from structural damage and thereby may account for maintaining muscle function (Anderson, 1987). Thus, different AChEIs can produce opposite effects on muscle contracture.

37.6 Muscle fiber histopathology

By using actomyosin ATPase reaction, analysis of untreated rat SOL revealed predominantly type 1 fibers with a few type 2A and 2B fibers. In contrast, EDL is composed predominantly of type 2 fibers with few type 1 fibers. The total fiber numbers are approximately 1800 in SOL and 2500 in EDL (Gupta et al., 1989).

OP pesticides and nerve agents and some carbamates in a single sublethal dose are known to cause fasciculations and induce myopathy (histopathological changes) in the diaphragm, SOL, EDL, sternomastoid, gastrocnemius, triceps, and tibialis of experimental animals. Pathological changes in skeletal muscles by AChE inhibitors are observed in the region adjacent to the motor endplate. Patterson et al. (1987) determined the necrotic lesions by assessing endplates in skeletal muscles of rats acutely intoxicated with DFP (1.5 mg/kg, s.c.). In SOL, these are found only in the midsection of the muscle, while in EDL, the endplates are found throughout the muscle

TABLE 37.5 Number of necrotic fibers following a single sublethal injection of DFP (1.5 mg/kg, s.c.).

Time after DFP administration	Soleus		EDL		Diaphragm	
	AChE	Lesions	AChE	Lesions	AChE	Lesions
Control	100%	0	100%	0	100%	0
1 day	24%	27 ± 6[a]	17%	64 ± 11[a]	13%	308 ± 58[a]
2 day	24%	39 ± 9[a]	14%	71 ± 12[a]	13%	333 ± 63[a]
3 day	39%	5 ± 1[a]	31%	49 ± 5[a]	25%	174 ± 35[a]
7 day	65%	0	53%	0	69%	0

Values of AChE activity are expressed as % remaining activity of control (100%); numbers of lesions are presented per cross section from the mid-belly region of muscle; data are presented as the means ± SEM ($n = 5–10$).
[a]*Significant difference between control and DFP-treated rats* ($P < .05$).

TABLE 37.6 Necrotic fibers in skeletal muscles of rats following a sublethal dose of soman, sarin, tabun, or VX.

Time (posttreatment)	Muscle	Number of lesions/1000 fibers			
		Soman (100 μg/kg, s.c.)	Sarin (110 μg/kg, s.c.)	Tabun (200 μg/kg, s.c.)	VX (12 μg/kg, s.c.)
1 h	DIA	0	9 ± 1	6 ± 2	26 ± 5
	SOL	0	12 ± 4	9 ± 2	16 ± 3
	EDL	0	0	0	0
6 h	DIA	3 ± 2	100 ± 30	48 ± 12	233 ± 45
	SOL	13 ± 3	111 ± 38	21 ± 4	97 ± 17
	EDL	0	0	0	23 ± 10
24 h	DIA	260 ± 48	435 ± 154	302 ± 32	322 ± 49
	SOL	48 ± 9	101 ± 24	66 ± 14	99 ± 20
	EDL	1 ± 0	103 ± 45	33 ± 5	28 ± 14
3 days	DIA	74 ± 24	490 ± 66	541 ± 31	305 ± 78
	SOL	9 ± 3	135 ± 42	192 ± 29	186 ± 37
	EDL	0	18 ± 17	66 ± 12	79 ± 16
7 days	DIA	0	216 ± 69	40 ± 10	75 ± 12
	SOL	0	237 ± 61	28 ± 4	62 ± 7
	EDL	0	30 ± 11	3 ± 1	27 ± 11

Value are means ± SEM of necrotic lesions ($n = 5$).

length. In EDL, the fibers are innervated at different levels throughout the length of the muscle, while in SOL, the nerve makes functional contact just in the midsection. No endplates or lesions are found outside this area. Gupta et al. (1985, 1986) found the highest number of lesions in all three muscles (diaphragm, SOL, and EDL) of rats within 24–48 h after a single injection of DFP (1.5 mg/kg, s.c.), when the inhibitory effect on AChE activity was also maximal (Table 37.5). The diaphragm muscle had the highest number of lesions, followed by the EDL and SOL.

Data on soman- (100 μg/kg), sarin- (110 μg/kg), tabun- (200 μg/kg), and VX = induced (12 μg/kg) pathological changes in skeletal muscles of rats are presented in Table 37.6. Following soman treatment, the number of necrotic fibers in the SOL and the diaphragm was found

to increase for up to 24 h, and no new lesions appeared thereafter. By day 7, these two muscles appeared to have fully recovered from the soman effect. In EDL, no morphological changes were seen at any time. While the acute toxic effects of tabun are more or less similar to soman, there are also some significant differences between these two agents. Unlike soman, tabun caused prolonged AChE inhibition and progressive development of muscle necrosis over a period of 3 days. While the primary toxic effect of tabun occurs due to inhibition of AChE activity, some of the differences seen in regard to other nerve agents may be due to additional actions of the cyanide group that is released during AChE inhibition. The inhibitory effect of CN on oxidative metabolism could result in reduced de novo synthesis, delaying synthesis of AChE, and prolonged inhibition of Ca^{2+} sequestration into the SR of skeletal muscles. Therefore, in the context of OP nerve agent-induced myopathy, soman, sarin, tabun, or VX caused the greatest number of lesions in the diaphragm, followed by the SOL, and then in the EDL during the first 24–48 h (Gupta et al., 1987a,b, 1991). With all four OP nerve agents, the greatest number of lesions occurred in animals exhibiting severe muscle fasciculations (Gupta et al., 1986, 1987a,b, 1991; Inns et al., 1990; Bright et al., 1991). By day 7, muscles recovered from soman-induced myopathy, but not from any other nerve agents since the necrotic lesions were still evident.

The earliest lesions are focal areas of abnormality in the subjunctional section of the muscle fiber adjacent to the motor endplate, including eosinophilia and sarcoplasmic swelling (Laskowski et al., 1977; Gupta et al., 1985, 1986; Patterson et al., 1987). Mitochondria are disrupted as evidenced by clumping of highly reactive material of lactate dehydrogenase (LDH) and reduced nicotinamide adenine dinucleotide reactions. These focal changes progress to a breakdown of subjunctional fiber architecture, loss of striations, and then phagocytosis. Longitudinal sections reveal that necrosis affects only a small segment of fiber lengths. During the latter stages, progressively greater lengths of muscle fibers are affected (Gupta et al., 1986; Patterson et al., 1987). Serial cross sections of 10 μm thickness indicate that the number of lesions correlates with the greatest density of endplates (Patterson et al., 1987). The longer the lag between injection and sacrifice, the greater the extent of the lesions. A significant increase in blood CK activity coincides with the appearance of myonecrosis, indicating destruction of the muscle membrane (Sket et al., 1989; Gupta et al., 1991, 1994).

Subjunctional changes in the muscle fibers, such as supercontraction of subjunctional sarcomeres, as well as disruption of cytoarchitectural organization, are always present (Gupta et al., 1986, 1987a,b, 1991; Inns et al., 1990; Bright et al., 1991). The initial changes are in the mitochondria which first swell and then show lysis of the central cristae. Myelin figures beneath the endplate are frequently observed, while the region more distal to the endplate is less affected. The nucleoli of the muscle cell nuclei are enlarged and move to the periphery of the nucleus. This myopathy can be induced with OP prototype DFP (Gupta et al., 1986; Misulis et al., 1987; Patterson et al., 1987, 1988; Sket et al., 1991a,b), or the OP nerve agents soman, sarin, tabun, and VX (Gupta et al., 1987a,b, 1991; Inns et al., 1990; Gupta and Dettbarn, 1992).

Laskowski et al. (1975) reported ultrastructural changes in the subsynaptic folds that were quite varied, even between muscle fibers from the same diaphragms of rats acutely treated with paraoxon. The fact that some endplates were totally degenerated after 2 days of paraoxon treatment, while others appeared almost normal even after 5 days, indicates that some endplates are more resistant than others. The most consistent change at the endplates was the presence of vesicular structures in the synaptic clefts. Some regions of the subsynaptic folds contained varying sizes of vacuoles and vesicles. There appears to be a wide variation in the severity of the lesions in the subsynaptic folds even in the same muscle. After 2 days of paraoxon treatment, the muscle surrounding the motor endplate often showed less cytoarchitectural organization than control muscles. Myelin figures beneath the endplate were frequently observed, while the region of muscle distal to the endplate was less severely affected.

Regardless of the diversity in OP structures, it is evident that induced myopathy is dose-dependent and rests on both a critical duration and degree of AChE inhibition. The higher number of necrotic lesions found with faster rates of AChE inactivation indicates the involvement of ACh in the generation of myopathy (Wecker et al., 1978a,b; Gupta et al., 1986, 1987a,b, 1991). Regardless of the OP involved, AChE inhibition of more than 80% for about 2 h is necessary to initiate severe muscle fiber necrosis. AChE inhibition resulting in an excess of ACh and its prolonged functional interactions with the nAChRs are responsible for producing lesions. However, some anticholinesterase (anti-ChEs) agents have been shown to interact directly with the nicotinic receptors (Tattersall, 1990, 1992). The OP compounds acting directly on postsynaptic membranes or on components of the muscle cell were also considered in the etiology of myopathy (Laskowski and Dettbarn, 1971). However, the prior injection of either tubocurarine or α-bungarotoxin can prevent development of the myopathy and indicates that neither the OP per se nor ACh by itself causes the damage; rather, the damage is due to changes in transmitter–receptor interaction. Tubocurarine and α-bungarotoxin, by occupying

the postjunctional receptors, prevent the ACh from interacting with the receptor. The primary defect is at both presynaptic and postsynaptic sites. This not only supports the concept of an abnormal neurotrophism as a mechanism of myopathy, but also allows the possibility that the primary defect is in the cholinergic system of nerve and muscle.

Evidence for the involvement of locally elevated levels of ACh was confirmed since denervation prevented myopathy or nAChR blockade with α-bungarotoxin (Wecker and Dettbarn, 1976; Dettbarn, 1984). It was clearly demonstrated that transection of the phrenic nerve to the rat hemidiaphragm prevented myopathic development, while in the contralateral innervated hemidiaphragm, the number of lesions increased. Thus, the common denominator is muscle hyperactivity, such as fasciculations (Gupta et al., 1986, 1987a,b, 1991; Adler et al., 1992). The longer the muscle hyperactivity lasts, the greater the number of necrotic muscle fibers found.

There is ample evidence that ACh accumulation is involved in causing Ca^{2+} influx into skeletal muscle fibers during anti-ChE poisoning. Experiments with sarin (25−150 μg/kg, s.c.) in mice revealed a similar role of Ca^{2+} in muscle fiber damage (Inns et al., 1990). Ca^{2+} increase was found in the diaphragm of those mice to which sarin had been administered at doses of 50 μg/kg or above. Ca^{2+} accumulation, which was confined to the region of the motor endplates, occurred earliest and remained the longest in the diaphragm from those animals receiving the highest doses of sarin. Generally, Ca^{2+} accumulation can be detected on day 1 after injection and none after day 7. This observation coincides with the duration of appearance and disappearance of muscle lesions. It was suggested that Ca^{2+} is among a series of events that ultimately lead to myonecrosis. Mitochondrial damage by excessive Ca^{2+} is expected to cause a partial or complete depletion of ATP synthesis and consequently excessive generation of oxygen- and nitrogen-free radicals and eventually myotoxicity (Gupta and Dettbarn, 1987, 1992; Gupta et al., 2001a,b, 2002). Taken together, following an acute exposure to an OP compound at a higher dose, a causal relationship exists between a critical level of AChE inhibition, Ca^{2+} accumulation, depletion of ATP, and appearance of muscle fiber necrosis.

Chronic exposure (30−60 days) of rats to paraoxon, at doses (0.05−0.1 mg/kg, s.c./day) that do not produce parasympathomimetic effects, led to necrosis of muscle fibers in diaphragm muscle, which was qualitatively similar to that following the administration of a single high dose of paraoxon (0.23 mg/kg, s.c.). The lesion was characterized by the presence of central nuclei, fiber splitting, and breakdown of fiber architecture followed by phagocytosis and necrosis. With either dose, AChE inhibition occurred in the endplate regions, not in the nonendplate regions (Wecker and Stouse, 1985).

37.7 Muscle cytotoxicity biomarkers

37.7.1 Creatine kinase and creatine kinase isoenzymes

CK catalyzes the synthesis of ATP and PCr in a reverse Lohmann reaction. Normal distribution of CK and its isoenzymes in the skeletal muscle of untreated control male Sprague-Dawley rats is shown in Table 37.7. The findings revealed that the fast-muscle EDL had the maximal CK activity, followed by diaphragm, with the lowest in the SOL. Electrophoretic separation of CK isoenzymes in all three muscles revealed the existence of only CK-MM isoenzyme. Further separation of CK-MM isoenzyme for subforms showed only CK-MM3 in all three muscles. Compared to muscles, serum had very little CK activity; however, the CK consisted of three distinct isoenzymes: CK-BB (15.3%), CK-MB (3.9%), and CK-MM (80.8%).

TABLE 37.7 Normal distribution of CK and CK isoenzymes in skeletal muscles and serum of rats.

	Total CK	CK isoenzymes		
		CK-BB (CK-1)	CK-MB (CK-2)	CK-MM (CK-3)
Soleus	2,062,800 ± 71,065 (100)	ND	ND	2,062,800 ± 71,065 (100)
EDL	4,659,125 ± 185,583 (100)[a,b]	ND	ND	4,659,125 ± 185,583 (100)[a,b]
Diaphragm	3,018,240 ± 110,777 (100)	ND	ND	3,018,240 ± 110,777 (100)
Serum	3769 ± 240 (100)	576 ± 45 (15.3)	148 ± 17 (3.9)	3086 ± 209(80.8)

Values expressed in terms of IU/L are presented as means ± SEM ($n = 4–6$); numbers in parentheses are percentages of isoenzymes to total CK activity (100%); ND, none detected.
[a] *Significant difference between EDL and soleus* ($P < .001$).
[b] *Significant difference between EDL and diaphragm* ($P < .001$).

Further electrophoresis of the serum CK-MM isoenzyme revealed the presence of three subforms: CK-MM1 (6.3%), CK-MM2 (24%), and CK-MM3 (69.7%) (Gupta et al., 1994). Literature abounds showing that the CK-MM3 subform secretes from muscles into the plasma, where it converts into the MM2 and MM1 subforms by carboxypeptidase-N2 (CPN-2).

Within 1 h of exposure to carbofuran (1.5 mg/kg, s.c.), CK activity was significantly reduced in the SOL, while it increased in the diaphragm. At the same time, activities of CK and all three CK isoenzymes were significantly elevated in serum. An important finding was that carbofuran or methyl parathion caused a shift in the serum CK-MM subform; that is, higher sequential conversions of CK-MM3 subform to CK-MM2 and CK-MM2 to CK-MM1, possibly due to enhanced CPN-2 activity (Gupta et al., 1994).

37.7.2 Lactate dehydrogenase and lactate dehydrogenase isoenzymes

LDH catalyzes the synthesis of lactate and pyruvate in a reversible reaction and is commonly used as a biomarker of cell damage or death. Normal distribution of LDH and its isoenzymes in skeletal muscles of rats revealed that, in controls, LDH activity was found to be highest in the EDL, followed by the diaphragm, and lowest in the SOL (Table 37.8). Compared to muscles, the enzyme activity was meager in serum. Interestingly, all three muscles and serum contained all five electrophoretically distinct LDH isoenzymes, although with varying quantities. Each muscle presented a characteristic LDH isoenzyme pattern. For example, EDL contained a large proportion of LDH-5 (62.6% of total activity) and very little LDH-1 (3.5%). SOL, on the other hand, had a very small amount of LDH-5 (11.3%) and about 20% each of the other four isoenzymes (LDH-1−LDH-4). In general, values of LDH isoenzymes in diaphragm were intermediate to the EDL and SOL. Diaphragm had predominantly LDH-5 and LDH-4 (40% and 27.7%, respectively). In control serum, isoenzyme LDH-5 was 87% of the total LDH activity (100%).

Total LDH activity was significantly enhanced in EDL, diaphragm, and serum by carbofuran (1.5 mg/kg, s.c.) or methyl parathion (5 mg/kg, i.p.) within 1 h of injection. Each AChE inhibitor caused a marked elevation of all five isoenzymes in serum, with maximum increases in LDH-1 and LDH-4 (threefold). Unlike serum, muscle LDH isoenzymes depicted variable patterns by carbofuran or methyl parathion intoxication. A significant decline in ATP appears to be the mechanism involved in leakage of cytoplasmic/mitochondrial enzymes into the circulation (Gupta et al., 1994).

37.8 Skeletal muscle involvement in tolerance development

Repeated application of OP nerve agents or pesticides for a prolonged period in concentrations that initially do not produce obvious symptoms of toxicity can lead to a limited degree of adaptation, as seen in a reduction of the duration and intensity of muscle fasciculations, muscle fiber necrosis, and behavioral tolerance (Wecker and Dettbarn, 1976; Gupta et al., 1985, 1986). Various cholinergic mechanisms in skeletal muscles underlying this phenomenon have been well-documented, including: (1) reduced cholinergic-binding sites of muscarinic and nicotinic receptors, (2) reduced uptake of choline, and (3) stimulation of AChE synthesis (Gupta et al., 1985, 1986). In an in vivo subchronic study (DFP, 0.5 mg/kg, s.c./day for 14 days) in male rats, Gupta and Dettbarn (1986) demonstrated complete recovery of protein synthesis in skeletal muscles (SOL, EDL, and diaphragm) during the tolerance phase (day 14) from inhibition of protein synthesis observed during the toxicity phase (day 5). Further, during the toxicity phase (day 5), inhibition of in vivo protein synthesis was comparable to that seen from an acute dose of DFP (1.5 mg/kg, s.c.). Additional mechanisms that may contribute to general tolerance are the availability of other serine active site enzymes, such as carboxylesterase (CarbE) and BuChE. Although the functional role of these enzymes is unknown, binding to and inhibition of these enzymes reduce the free concentration of inhibitors that otherwise would have been available to interact with AChE (Gupta et al., 1985). This is supported by studies showing that the toxicity of OPs can be potentiated by inhibition of CarbE, and tolerance to soman develops when plasma CarbEs recover during chronic exposure (Sterri et al., 1981).

A major mechanism causing adaptation to the necrotic action of DFP at the NMJ is the reduction of nAChRs (B_{max}, 56%) without significant change in affinity constant (K_D). This loss of receptors can also explain the disappearance of fasciculations that was observed between days 3 and 5 of treatment (Gupta et al., 1986). Whether presynaptic receptors regulating fasciculations are involved in this process remains to be determined. Changes in the postsynaptic receptor density could occur as an adaptation mechanism of the cell to the excessive cholinergic stimulation caused by AChE inhibition. The loss of ACh receptors may be caused by a reduction of junctional folds (Laskowski et al., 1975) on which most of the nicotinic ACh receptors are located. Changes in the ionic milieu, especially increases in Ca^{2+} caused by the increased neuromuscular activity during DFP-induced fasciculations, may be involved in the loss of secondary junctional folds. In essence, at the NMJ, the two major mechanisms leading to adaptation are: (1) an increased

TABLE 37.8 Normal distribution of LDH and LDH isoenzymes in skeletal muscles and serum of rats.

	Total LDH	LDH isoenzymes				
		LDH-1 HHHH	LDH-2 HHHM	LDH-3 HHMM	LDH-4 HMMM	LDH-5 MMMM
Soleus	72,720 ± 2484 (100)	14,962 ± 476 (20.7)	19,054 ± 617 (26.3)	16,118 ± 692 (22.2)	14,254 ± 1332 (19.5)	8316 ± 890 (11.3)
EDL	207,300 ± 22,945 (100)[a,b]	7290 ± 1890 (3.5)[a,b]	12,300 ± 2033 (5.9)[a,b]	18,105 ± 1498 (8.7)	40,420 ± 4100 (19.5)[a,b]	129,870 ± 15,298 (62.6)[a,b]
Diaphragm	129,120 ± 4828 (100)	17,020 ± 504 (13.2)	17,498 ± 571 (13.5)	16,190 ± 780 (12.6)	26,738 ± 1080 (27.7)	51,680 ± 3081 (40.0)
Serum	740 ± 28 (100)	9.8 ± 1.4 (1.3)	17.4 ± 2.0 (2.3)	10.2 ± 1.2 (1.4)	38.0 ± 3.8 (5.2)	643 ± 65 (87.0)

Values expressed in terms of IU/L are presented as means ± SEM ($n = 4–6$); numbers in parentheses are percentages of isoenzymes to total LDH activity (100%).
[a]*Significant difference between EDL and soleus* ($P < .01$).
[b]*Significant difference between EDL and diaphragm* ($P < .01$).

recovery of AChE activity as a result of de novo synthesis (Gupta et al., 1986), (2) a reduction in nAChR binding sites (Gupta et al., 1985, 1986, 1987a,b), and (3) modification of ACh release from presynaptic sites (Carlson and Dettbarn, 1988).

37.9 Skeletal muscle involvement in intermediate syndrome

OP insecticide-induced IMS was reported for the first time in human patients in Sri Lanka in 1987 (Senanayake and Karalliedde, 1987; Karalliedde and Henry, 1993). Thereafter, this syndrome has been reported in South Africa (1989), Turkey (1990), Belgium (1992), India (2003), and many other countries. IMS is clearly a separate entity from acute cholinergic crisis and delayed neuropathy. IMS is a life-threatening complication of OP poisoning, which most commonly occurs 48−72 h after exposure. Thus far, IMS has not been documented with OP nerve agents. Clinically, IMS is characterized by acute respiratory paralysis and weakness in the territories of several cranial motor nerves, neck flexors, and facial, extraocular, palatal, nuchal, and proximal limb muscles. Despite severe AChE inhibition, muscle fasciculations and muscarinic receptor-associated hypersecretory activities are absent. Based on EMG findings from OP-poisoned patients and experimental studies on laboratory animals, it has been found that the defect in IMS is at the neuromuscular endplate and postsynaptic levels, but the effects of neural and central components in muscular weakness have not been ruled out. EMG in the early stages reveals marked decrements at low rates of repetitive nerve stimulation and increments at a high rate, suggesting diverse types of impaired neuromuscular transmission. IMS seems to be due to persistent AChE inhibition at the endplate, presumably leading to combined presynaptic and postsynaptic impairment of neuromuscular transmission. Very little is known about the type of damage at the motor endplate or about risk factors associated with IMS. For details on the involvement of muscles in IMS, see De Bleecker (2006).

37.10 Prevention/treatment of myopathy

Various pharmacologic and therapeutic drugs have been tested to prevent or treat myopathy. Drugs effective in the treatment of neuromuscular signs of anti-ChE toxicity include: (1) oximes that reactivate the phosphorylated AChE (Thiermann et al., 2005; Marrs and Vale, 2006), (2) subparalyzing and paralyzing doses of *d*-tubocurarine (Clinton and Dettbarn, 1987; Patterson et al., 1987), (3) atropine sulfate/atropine methyl nitrate (Patterson et al., 1987; Clinton et al., 1988), (4) diazepam, and (5) creatine phosphate (Clinton et al., 1988). In several studies, reversible AChE-inhibiting carbamates, atropine, or anticonvulsants were given with oxime to achieve an optimal effect. Some of the drugs, including AChE reactivators, muscarinic and nicotinic ACh receptor blockers, NMDAR antagonists, anticonvulsants, and antioxidants, are described here briefly in terms of their prophylactic/therapeutic efficacy. It should be noted that some of these agents, however, produce toxicity when given for an extended period of time, thereby limiting their utility.

37.11 Acetylcholinesterase reactivators and acetylcholinesterase receptor blockers

The standard treatment of nerve agent-induced muscle toxicity calls for reactivation of the phosphorylated AChE with an oxime. Oximes such as obidoxime [bis(4-formylpyridiniomethyl) ether dioxime, also known as Toxogonin], pralidoxime (2-pyridine aldoxime methochloride/2-PAM), and a few others have been found very effective when given in combination with other drugs such as atropine, *d*-tubocurarine, and diazepam. The effectiveness of pretreatment with oximes varies with the chemical structures of the nerve agents and depends on the time after exposure. For example, it has been established that asoxime chloride (HI-6) effectively reactivates AChE inhibited by soman, while TMB-4 is known as one of the most efficient reactivators of tabun-inhibited enzyme. In in vivo studies, Jovanovic (1983) evaluated the effects of two bis-pyridinium oximes (BDB-27 and HGG-12) on neuromuscular blockades induced by nerve agents (sarin, soman, tabun, and VX) in rats, and the effectiveness was compared with the two most potent oximes (HI-6 and TMB-4). It was found that BDB-27 was equal or superior to HI-6 in sarin, soman, and VX, and to TMB-4 in tabun-poisoned animals. The potency of HGG-12 was equal to HI-6 only in soman poisoning, but it was much less pronounced against neuromuscular blockades induced by the other nerve agents. In general, oxime therapy becomes progressively ineffective with time due to changes in the enzyme−inhibitor complex, which loses an alkyl radical. This so-called aged phosphorylated enzyme complex is resistant to oxime reactivation (Radic and Taylor, 2006). Thus, prevention of formation of the enzyme−inhibitor complex seems to be one of the preferred strategies in the treatment of myopathy.

The best pretreatment should include agents that prevent access of the nerve agent to AChE without affecting its activity, or a combination of drugs that would decrease the release of ACh from nerve terminals and partially

block access to the nicotinic and the presynaptic and postsynaptic muscarinic receptors. So far, timely administration of atropine sulfate or atropine methyl nitrate, in combination with an oxime (such as 2-PAM) or diazepam, is currently the treatment of choice for OP poisoning (Marrs and Vale, 2006). Atropine modifies ACh receptor interaction at the NMJ postsynaptically by shortening the opening time of the ion channel, and possibly by lowering Na^+ conductance. Atropine sulfate has been observed to reduce motor activity and muscle necrosis caused by AChE inhibitors (Clinton and Dettbarn, 1987). Atropine methyl nitrate is four times more potent than atropine sulfate in its peripheral effects and has been confirmed to ameliorate peripheral motor activity caused by AChE inhibitors (Clinton et al., 1988). Atropine methyl nitrate (16 mg/kg, s.c.) failed to ameliorate the central effects of both soman and DFP due to its quaternary structure that hinders its passage across the blood–brain barrier (Clinton et al., 1988).

Earlier studies with *d*-tubocurarine and atropine indicated that nicotinic and muscarinic ACh receptor blockers can modulate the release of ACh induced by an inhibitor of AChE (Carlson and Dettbarn, 1988). The effects of these drugs, whether inhibiting or stimulating ACh release, are concentration dependent and determined by the frequency of nerve activity (Bowman, 1980; Wessler et al., 1987a,b). Therefore, drugs that reduce axonal hyperexcitability by decreasing the amount of ACh released from the nerve terminals without interfering with normal transmission, provide another pretreatment possibility. Protection against DFP-induced myopathy was achieved using small concentrations of atropine sulfate (16 mg/kg, s.c.) or atropine methyl nitrate (16 mg/kg, s.c.) and *d*-tubocurarine (75 μg/kg, s.c.) that prevented fasciculations and muscle necrosis without interfering with normal neuromuscular function (Patterson et al., 1987). In this study, pretreatment agents acted presynaptically by preventing DFP-induced backfiring and muscle fasciculations, possibly by reducing the release of ACh. The protective drugs in the concentrations used had no significant effect on the normal characteristics of conduction and transmission. Carlson and Dettbarn (1988) showed that *d*-tubocurarine or atropine sulfate in subparalytic concentrations prevented the increases in MEPP frequency when given prior to an AChE inhibitor, or attenuated the increased frequency to normal when given after the increase of frequency. At a subparalytic dose *d*-tubocurarine (50 μg/kg, i.v.) is known to suppress fasciculations by preventing repetitive firing of nerve terminals. Furthermore, *d*-tubocurarine and atropine sulfate reduced Ca^{2+} influx and ACh release (through presynaptic receptors), which is associated with repetitive activity, as seen during AChE inhibitor-induced antidromic firing.

37.11.1 *N*-Methyl-D-aspartate receptor antagonist

In a series of in vivo experiments, rats receiving a sublethal dose of soman (100 μg/kg, s.c.), sarin (110 μg/kg, s.c.), tabun (200 μg/kg, s.c.), VX (12 μg/kg, s.c.), or DFP (1.5 mg/kg, s.c.) developed seizures and severe muscle fasciculations within 15–20 min that lasted for about 4–6 h. Marked inhibition of AChE activity and necrotic lesions in skeletal muscles (SOL, EDL, and diaphragm) became evident between 1 and 24 h postinjection and persisted for several days. Pretreatment of rats with a NMDAR antagonist memantine HCl (18 mg/kg, s.c.), given in combination with atropine sulfate (16 mg/kg, s.c.) 60 and 15 min prior to DFP or nerve agents, respectively, significantly attenuated AChE inhibition and prevented myonecrosis and muscle fasciculations, as well as other signs of behavioral toxicity. No muscle fasciculations were seen at any time (Gupta and Dettbarn, 1992).

Protection of AChE inhibition was greater when memantine was given prophylactically than therapeutically. It is noteworthy that memantine itself does not influence normal activity of AChE in non-OP-treated animals. Although the precise mechanism involved in reduction of AChE inhibition by memantine against nerve agents is yet to be elucidated, spontaneous reactivation as the cause of the remaining high-enzyme activity was ruled out since the enzyme activity was determined within a short period of time; that is, 1 h after acute intoxication with soman, sarin, tabun, VX, or DFP (Gupta et al., 1986, 1987a,b, 1991; Gupta and Dettbarn, 1992), and AChE activity remained low when only atropine sulfate (16 mg/kg, s.c.) was used. McLean et al. (1992) revealed that memantine did not prevent inhibition of AChE by edrophonium, an anionic site inhibitor, or by decamethonium, a peripheral site inhibitor. Thus, memantine appears to bind to a different modulatory site to protect this enzyme's activity. Stojiljković et al. (2019) described that the mechanism of the antidotal effect of memantine against soman poisoning appears to be a combination of AChE protection and NMDA receptor-blocking action. The other mechanisms by which memantine might have attenuated AChEI's toxicity may include the following:

- Reduced reflex excitability of both flexors and extensors (Wand et al., 1977);
- Reduced high frequency of repetitive activation of peripheral nerves by reducing the permeability of Na^+ and Ca^{2+} in axonal membranes (Wesemann and Ekenna, 1982; Wesemann et al., 1983);
- Blockage of nicotinic ACh receptor–ion channel complex (Masuo et al., 1986);

- Prevention of neural hyperexcitability (McLean et al., 1992);
- Central muscle relaxation (Grossmann and Jurna, 1977);
- Reduced seizures by uncompetitive NMDAR blockage (Danysz et al., 1994; Carter, 1995; Parsons et al., 1999; Stojiljkovic et al., 2019);
- Prevention of cellular energy depletion (Gupta and Goad, 2000);
- Prevention of oxidative/nitrosative stress (Gupta et al., 2005).

Although not all of these mechanisms have been investigated in the protection of OP nerve agent-induced myopathy by memantine, it appears that memantine provides protection by multiple mechanisms. It needs to be mentioned that no significant change occurred in AChE activity in skeletal muscles of OP-untreated rats receiving memantine and atropine sulfate. Prophylactic administration of memantine and atropine sulfate also blocked the AChEI-induced increase in levels of citrulline and F_2-isoprostanes, markers of NO synthesis and lipid peroxidation, respectively (Milatovic et al., 2005). Memantine has the advantage of providing prophylactic benefits without producing sedation or any other side effects. Thus, memantine may prove to be a superior drug to many other agents.

37.11.2 Anticonvulsants and anesthetics

The prevention and treatment of OP-induced myopathy are both complex since individual AChEIs differ in their major sites of action. As mentioned earlier, soman-produced muscle hyperactivity was generated mainly in the CNS, while DFP-induced hyperactivity arose approximately equally from the CNS and the peripheral nervous system and NMJ. Clinton et al. (1988) reported that in the case of both soman (90 μg/kg, s.c.) and DFP (1.5 mg/kg, s.c.) poisoning, ketamine (25 mg/kg, s.c.) reduced centrally generated motor activity, while atropine methyl nitrate (16 mg/kg, s.c.) and sodium phenytoin (15 mg/kg, i.v.) had no significant effect. It was suggested that the effectiveness of ketamine may alter patterns of neuronal firing by reducing high-frequency neuronal discharges that are characteristic of seizures (MacDonald and McLean, 1986). Ketamine is known to act on sodium channels by producing a dose-dependent reduction of inward sodium current in a manner similar to that of local anesthetics, with a resultant suppression of high-frequency neuronal bursts, which are manifested as excess muscle activity seen with OP poisoning. Since ketamine and drugs in its class also have specific anticholinergic effects at both nicotinic and muscarinic (more potent at nicotinic than at muscarinic) receptors (Kloog et al., 1977), it may be a direct antagonist in the CNS against the cholinergic activation caused by AChEIs (Gupta et al., 1987a,b). Additionally, ketamine can inhibit excitation–contraction coupling within the muscle by altering Na^+ conductance at the muscle membrane (Marwaha, 1980a,b).

The anticonvulsant phenytoin did not reduce central motor activity induced by either soman or DFP (Clinton et al., 1988). Phenytoin suppresses sustained high-frequency neuronal firing through a membrane potential-dependent blockade of Na^+ channels with resulting inhibition of nonsynaptic events involved in epileptogenesis (MacDonald and McLean, 1986; Yaari et al., 1986). The failure of phenytoin to control the seizures induced by AChEIs suggests that they may not result from the same mechanisms of epileptogenesis as seizures produced by maximal electrical shock, against which phenytoin is most effective. Conventional anticonvulsant compounds have been reported to provide limited protection against nerve agent-induced seizures and muscle necrosis when given therapeutically (Lipp, 1972; Clinton et al., 1988). Sedation, tolerance, and abuse potentially limit prophylactic use of benzodiazepine compounds, however.

In animal models, clonidine (an alpha2 adrenergic agonist) has also been reported to prevent nerve agent-induced seizures (Buccafusco et al., 1988). Prophylactic use of clonidine may be limited by the marked ataxia and sedation produced by this drug in effective concentrations.

37.11.3 Antioxidants, spin-trapping agents, and creatine

Scavenging or prevention of the ROS generation originated through various excitotoxicity mechanisms are of particular interest. An antioxidant such as the lipid-soluble vitamin E (α-tocopherol) is an excellent blocker of ROS production, as it extracts hydrogen and interrupts lipid peroxidation. Vitamin E mainly acts as a chain-breaking antioxidant and radical scavenger, protecting cell membranes against oxidative damage. There are reports demonstrating that vitamin E concentrates in the mitochondria, the major site for the generation of ROS, as well as energy metabolites. Therefore, vitamin E regulates ROS production, maintains oxidative phosphorylation in mitochondria, and accelerates the restitution of high-energy metabolites.

A synthetic spin-trapping agent such as phenyl-*N*-*tert*-butylnitrone (PBN) is capable of scavenging many types of free radicals. This compound is widely used to trap

TABLE 37.9 Protective effect of PBN against DFP-induced fasciculations, AChE inhibition, and necrosis in rat EDL.

	Control	DFP[a]	PBN + DFP[b]	DFP + PBN[c]
Fasciculations	−	+	−	+
Necrotic fibers/cross section of muscle	2 ± 2	82.55 ± 2.98*	0**	67.0 ± 4.59
AChE activity	12.17 ± 0.73 (100%)	3.70 ± 0.35* (30%)	10.85 ± 0.59** (89%)	2.97 ± 0.17 (24%)

Activity of AChE is expressed in terms of nmol/mg protein/min and number of necrotic fibers/1000 muscle fibers. Ranking of fasciculations: −, absent; +, high frequency affecting all muscles. Values are means ± SEM of five muscles.
*Significant difference between control and DFP-treated rats ($P < .01$).
**Significant difference between DFP-treated rats and PBN + DFP-treated rats ($P < .01$).
[a]*DFP (1.7 mg/kg, s.c.) was given 1 h before sacrificing the rats.*
[b]*PBN (300 mg/kg, i.p.) was given 30 min before DFP (1.7 mg/kg, s.c.).*
[c]*PBN (300 mg/kg, i.p.) was given 20 min after DFP (1.7 mg/kg, s.c.) administration.*

ROS in a variety of physical, chemical, and biological studies using electron magnetic resonance spectrometry. PBN is known to be concentrated in the mitochondria, where it reacts with ROS and forms stable adducts, thereby maintaining normal levels of energy metabolites. In addition, PBN has other pharmacological actions, such as: (1) reversible Ca^{2+} channel blockade (Anderson et al., 1993), (2) direct reversible interaction with AChE against phosphorylation by DFP (Zivin et al., 1999; Milatovic et al., 2000a,b), and (3) protection of COX activity (Milatovic et al., 2001).

Pretreatment with PBN (75, 150, or 300 mg/kg, i.p.) 30 min prior to DFP injection (1.7 mg/kg, s.c.) in a dose-dependent manner protected AChE activity from inhibition, and prevented muscles from undergoing necrosis and rats from fasciculations (Milatovic et al., 2000a,b) (Table 37.9). The protective mechanism of PBN is proved to take place due to its ROS/RNS scavenging property (Gupta et al., 2000a, 2001b). Treatment with PBN 20 min after DFP exposure neither prevented fasciculations nor protected AChE activity. While the role of PBN as an antioxidant is well established, its prophylactic effect against excitotoxicity induced by an AChEI is due to its protection of AChE from critical inhibition (Milatovic et al., 2000a,b). Unlike PBN, vitamin E neither prevented DFP-induced muscle fasciculations nor protected AChE. Both vitamin E and PBN concentrate in the mitochondria, so they can regulate ROS production, maintain oxidative phosphorylation, and accelerate restitution of high-energy metabolites (Gupta et al., 2001a,b).

Since the major energy sources are ATP and PCr, an increase of both compounds in muscles through administration of creatine appears to sustain ATP levels under stress conditions. This is supported by the findings that rats pretreated intravenously with PCr showed reduced muscle necrosis that otherwise would have been seen following DFP treatment. PCr did not attenuate the DFP-induced muscle fasciculations that generated the necrosis (Clinton and Dettbarn, 1987).

37.12 Concluding remarks and future directions

Skeletal muscles are the target of a variety of chemicals, especially OP nerve agents. These agents modulate structural and functional properties of the muscles, and the toxic effects can range from minor chest pain, muscle cramps in the legs, complete paralysis, and even death. Both in vivo and in vitro data strongly implicate that OP nerve agents initially exert acute toxicity by excitotoxicity involving not only the cholinergic system, but also processes associated with noncholinergic mechanisms. These processes include the glutamatergic system, excess generation of free radicals (ROS/RNS), and alterations in antioxidants and the scavenging system, causing oxidative stress, lipid peroxidation, high-energy phosphate depletion, and muscle necrosis. Wide variations exist between slow and fast fiber-containing muscles, and they further appear to respond differently to each OP nerve agent. Available sensitive biomarkers are indicative of cytotoxicity and mitochondrial toxicity (Gupta et al., 2019). Myopathy induced by AChE-inhibiting OP nerve agents is a serious concern, since it is untreatable in most circumstances. Instead, prevention seems a better option. More potent AChE reactivators need to be developed for better therapeutic efficacy against OP nerve agent-induced myopathy.

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Chapter 38

Dermal toxicity of sulfur mustard

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38.1 Introduction

Skin integrity is in part determined by the interaction of a number of proteins that form a continuum of molecules linking together to ensure the epidermis and dermis are tightly attached to one another. These macromolecules include the keratins that form the intermediate filaments in the cytoplasm of keratinocytes, integrins which are made of two subunits and found in the hemidesmosomes of the basal keratinocyte membrane, and a variety of laminins, collagens, and their receptors, situated both in the basement membrane that separates the epidermis from the dermis, and in the dermis itself. Any chemical agent that disrupts this continuum of linking proteins will result in a breach in skin integrity and ensuing histopathology (Uitto et al., 2007). One such compound is the alkylating agent sulfur mustard [bis(2-chloroethyl) sulfide; SM], which causes detachment of the epidermis from the dermis. Skin exposure to SM starts a complex series of events with a host of normal skin responses to wounding which interact with, influence, and regulate each other to result in cutaneous toxicity. Various mediators of injury that regulate inflammation, immune responses, cell death, and a number of signaling pathways have been implicated in the process. This chapter describes our current knowledge of the cutaneous actions of SM, discussing the basic mechanism of action and mediators involved, to provide for the reader a comprehensive understanding of the histopathology of SM-exposed skin. The injury process is described, and SM-induced skin injury is compared to other types of wound injury. Additionally, various skin-injury models and potential therapeutic countermeasures are discussed.

38.2 Background

38.2.1 Military use

Sulfur mustard, a vesicant or blistering agent, has been used intermittently as a chemical warfare agent since 1917 when Germany first introduced it as a weapon against British soldiers in Ypres, Belgium. Subsequently in the 1930s it was reported as having been used by Italy against Abyssinia (Ethiopia), Poland against Germany, and Japan against the China. During the 1960s, SM was allegedly used by Egypt against Yemen. The last well-documented use in the 1980s was by Iraq against Iran and the Kurds (Papirmeister et al., 1991c). Noteworthy is the completed destruction of Libya's sulfur mustard stockpile in 2013, as overseen by the Organization for the Prohibition of Chemical Weapons (OPCW) and the United Nations. Recently however, chemical weapons, including sulfur mustard, have been used in the ongoing (2011−present) Syrian civil war.

38.2.2 Wound repair

Repair of the simplest skin wound requires the coordination of a number of physiological processes and events. The tissue injury causes blood vessel disruption and an activation of the inflammatory pathway that releases a battery of mediators. Reepithelialization requires activation of additional pathways and the initiation of specific events at specific times, such as extracellular matrix (ECM) remodeling and the proliferation and migration of keratinocytes. If these events are not precisely coordinated, the end result can be delayed healing and/or scar

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formation. Since the repair process is complicated, a review of the wound-healing events in dermal injury might be helpful. There are at least three phases to wound healing following dermal injury (Gurtner et al., 2008). (1) Inflammation (first 24–48 h): this phase occurs early and involves activation of the coagulation cascade, inflammatory pathways, and immune system. These biological processes involve an attempt to maintain homeostasis by preventing blood and fluid loss, removing dead tissue, and preventing infection. (2) Wound repair phase (2–10 days after injury): this phase involves the proliferation and migration of several cell types. Keratinocytes migrate over the injured dermis, endothelial cells proliferate to form new blood vessels, and fibroblasts differentiate into myofibroblasts in order to initiate wound contraction. (3) Remodeling phase (2–3 weeks postinjury): this phase results in the final wound repair. All the activated cellular processes turn off. Many cell types undergo apoptosis or leave the wound site. The ECM is remodeled and resumes normal functions.

These same basic phases are applicable to injury caused by SM, but the pathology frequently is more severe than other types of dermal wounds and the basic timing of the various phases may be extended in SM injury (Papirmeister et al., 1991b; Dacre and Goldman, 1996; Balali-Mood and Hefazi, 2006). Normal clothing provides little protection to the skin from a liquid or vapor exposure to SM. Once in contact with the skin, the lipophilic properties of SM allow it to rapidly penetrate the epidermal barrier, while its high reactivity and bifunctional nature lead to alkylation of a wide range of molecules (Fig. 38.1). The ultimate injury caused by SM is highly dependent on the dose and length of exposure, resulting in a wide range of histopathology. Erythema, resembling sunburn, may be the mildest and earliest form of skin injury, occurring 2–48 h post-SM exposure (USAMRICD, 2007). The erythema may be accompanied by pruritus or burning pain and small vesicles may eventually develop which coalesce to form bullae (Fig. 38.2). Fluid-filled blisters increase in size, their color ranging from yellowish to tan. The fluid itself does not contain active alkylating agent, nor does it have vesicating properties. When SM is applied as a liquid, the exposure concentration is even higher than a vapor application and severe lesions are more likely. The resultant wounds are prone to secondary infections and may cause chronic ulcers that are resistant to wound repair. There may be permanent pigmentation changes in the skin after exposure to SM. Clearly, the effects of SM on skin are complex and involve many systems and pathways. The following sections address the mechanism of action of SM and the systems and pathways involved in an organized way.

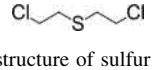

FIGURE 38.1 Chemical structure of sulfur mustard [bis(2-chloroethyl) sulfide].

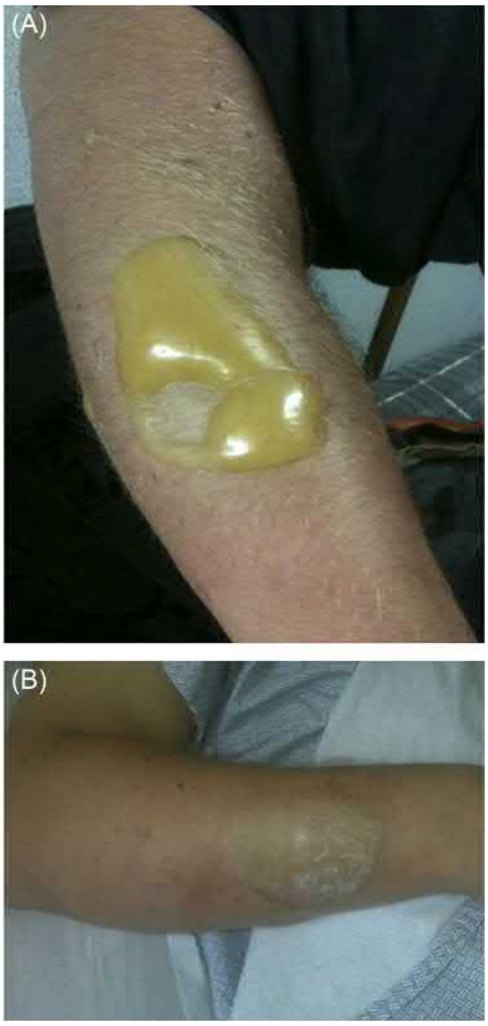

FIGURE 38.2 Fluid-filled bullous blister of a clam fisherman accidentally exposed to sulfur mustard, shown 24 h (A) and 6 days (B) postexposure. The patient was exposed when handling discarded WWI munitions trawled from the sea bed off the coast of New England in 2010 (Weibrecht et al., 2012). *Reprinted from Weibrecht, K., Rhyee, S., Manuell, M.E., et al., 2012. Sulfur mustard exposure presenting to a community emergency department. Ann. Emerg. Med. 59 (1), 70–74, with permission from Elsevier.*

38.3 Pathogenesis

The bulk of what is known about human skin injury from SM has been gathered from victims of the military use of the compound (Requena et al., 1988; Balali-Mood and Hefazi, 2006; Hefazi et al., 2006). Nevertheless, there are several reports where the cutaneous vesicating effects of SM were studied experimentally in human subjects (Papirmeister et al., 1991b; Dacre and Goldman, 1996).

In one comparative study (Daily et al., 1944) 12 volunteers had forearm vapor cup applications of SM, trifunctional nitrogen mustard (HN3), and lewisite. The conclusions were that SM exposure caused the largest vesicles formed, the earliest peak vesication, the longest healing time, and the most severe final scar. Lewisite was the second most potent agent and HN3 the least potent, although the HN3 lesions had the most edema of the three vesicants examined. The results confirmed the previously published studies which followed the development of skin pathogenesis after topical exposure to SM.

Clinical injury by SM has been extensively reviewed and will be summarized here. Sulfur mustard injury to human skin begins almost immediately after exposure when the highly reactive bifunctional SM directly alkylates resident proteins. However, recognizable skin pathology does not usually occur for several hours to a day after exposure. The very first physical sign of SM exposure is usually erythema which may or may not be associated with itching (Papirmeister et al., 1991b). This is often followed later by distinct fluid-filled blisters that break open and become covered with a scab. Many researchers have noted that the blisters resemble those formed by epidermolysis bullosa (EB), a genetic or acquired skin pathology that results in a separation of the epidermis from the dermis (Chang et al., 2018a). In both SM exposure and EB, there may be multiple rounds of blistering and healing in an individual (Balali-Mood and Hefazi, 2005; Pillay, 2008). In fact, some of the therapeutic agents for EB are being tested as potential medical countermeasures against SM injury. These will be addressed later.

38.3.1 Cytotoxicity of sulfur mustard

While stable in lipophilic solvents, SM has a half-life of only 24 min at room temperature in aqueous physiological solutions since it rapidly reacts with water to form thiodiglycol and HCl. This rapid activation of SM in an aqueous environment also allows it to react with small molecules of biological interest, as well as proteins, carbohydrates, lipids, RNA, and DNA (Bartlett and Swain, 1949; Papirmeister et al., 1991a; Debouzy et al., 2002; Noort and van der Schans, 2002). The initial reaction involves the formation of a cyclic ethylene sulfonium ion which readily targets reactive groups on skin components, including sulfhydryls, phosphates, ring nitrogens, and carboxyl groups. As a bifunctional alkylating agent, SM forms monofunctional adducts and intra- and intermolecular cross-links. Since it is so reactive and indiscriminate in its molecular targets, SM affects many pathways and is cytotoxic on many levels. There are at least three independent mechanisms of cytotoxicity that have been proposed and each will be addressed in separate sections.

38.3.1.1 Alkylation of DNA/poly(ADP-ribose) polymerase activation

A major target of SM alkylation is DNA, with which it can form both monofunctional adducts and bifunctional cross-links (Papirmeister et al., 1991a; Debiak et al., 2009), which can persist in affected skin for at least 21 days (Batal et al., 2013). A DNA damage response, initiated by the kinases ATM, ATR, and DNA-dependent protein kinase leads to cell cycle arrest (Lin et al., 1996; Long et al., 2016; Blackford and Jackson, 2017) allowing for a variety of DNA repair processes to ensue. Although the monofunctional adducts, typically at the N7 position on guanine, are a more prevalent consequence of SM exposure, DNA cross-links, especially between complementary strands, are most cytotoxic (Walker, 1971; Jowsey et al., 2012; Batal et al., 2013). The myriad pathways available to remove adducts and repair strand breaks produced by SM include both nucleotide excision repair (NER) and base excision repair (Matijasevic and Volkert, 2007; Jowsey et al., 2009), as well as those pathways more specific for double-strand breaks: nonhomologous end joining (NHEJ), homologous recombination (HR), and the alternate endjoining mechanisms, microhomology-mediated end joining and single-strand annealing (Sallmyr and Tomkinson, 2018). While rates and fidelity of repair differ among these pathways, and their dominance varies according to many factors, including the species and phase of the cell cycle, current information suggests that HR is perhaps the most important mechanism for sulfur mustard exposure, with NER and NHEJ pathways playing a supportive role (Jowsey et al., 2012).

SM-induced DNA damage also triggers activation of a family of nuclear repair enzymes called poly(ADP-ribose) polymerases (PARPs) (Papirmeister et al., 1985; Shall and de Murcia, 2000). While low levels of PARP activation may signal repair, excessive activity can deplete cells of PARP's required cofactor, NAD^+. Depletion of NAD^+ in turn inhibits ATP production, which is essential for metabolism (Martens and Smith, 2008). Apoptosis or necrosis may result, depending upon the level of ATP depletion, cell type, and other factors (Rosenthal et al., 2001). Preventing the depletion of NAD^+ by interfering with PARP activation has been the rationale for testing PARP inhibitors as therapeutic countermeasures (Debiak et al., 2009). Recent studies confirm that SM induces poly-ADP ribosylation in the HaCat keratinocyte cell line (Liu et al., 2016; Mangerich et al., 2016). Furthermore, ABT-888, a clinically relevant PARP inhibitor, reverses NAD^+ depletion produced by SM in these cells and reduces SM-induced edema in the mouse ear vesicant model (MEVM) pointing to the potential utility of selective PARP inhibitors in SM-induced injury (Liu et al., 2016).

38.3.1.2 Reactions with glutathione/oxidative stress

There is considerable evidence to support the hypothesis that oxidative stress plays a key role in the cutaneous vesicating actions of SM. Under homeostatic conditions, a net reducing environment is maintained in tissues by the presence of glutathione (GSH), which serves as a buffer against cytotoxic electrophiles and reactive oxygen species (ROS). Excess H_2O_2 is eliminated by glutathione peroxidase at the expense of GSH. Depletion of intracellular GSH allows the accumulation of oxidants, including H_2O_2, which become abundant and actively contribute to lipid peroxidation and other types of cellular damage. Dermatotoxic agents such as ultraviolet radiation (UVA) and psoralens have been shown to deplete intracellular GSH, resulting in cellular toxicity (Wheeler et al., 1986). The propensity of SM to react with sulfhydryls is thought to lead to a concentration-dependent depletion of reducing equivalents within cells. Electron paramagnetic resonance studies have suggested that SM and related vesicants can interact with key intracellular reductases to produce mustard-free radicals as an intermediate to ROS generation (Brimfield et al., 2009, 2012). In other tissues, SM exposure has been shown to reduce GSH, glutathione peroxidase, and glutathione reductase activity, potentially contributing to the depletion of reduced glutathione (Husain et al., 1996; Jafari, 2007; Pohanka et al., 2011, 2013). In addition, inflammatory cells, which infiltrate into the skin in response to SM-induced injury, generate additional ROS that contribute to oxidative stress (Droge, 2002). This information raises the possibility that oxidative stress plays a role in the vesicating actions of mustard alkylating agents.

Several reports more directly support the notion that GSH depletion and oxidative stress are important mechanisms of cutaneous toxicity induced by SM and its vesicating analogs. Cutaneous glutathione peroxidase gene expression is increased following SM treatment in the MEVM, suggesting that this enzyme is important in reducing oxidative stress due to SM exposure (Buxton et al., 2001). Treatment with the monovalent mustard analog, 2-chloroethyl ethyl sulfide (CEES), has been shown to significantly deplete GSH in mouse and human keratinocyte cell lines (Tewari-Singh et al., 2011). Furthermore, depletion of intracellular-reduced glutathione by buthionine sulfoxamine sensitizes keratinocytes treated in vitro with SM (Simpson and Lindsay, 2005) or CEES (Tewari-Singh et al., 2011).

There is more direct evidence from both in vivo and in vitro studies that CEES produces oxidative stress. CEES application to the dorsal skin of SKH-1 hairless mice led to time- and dose-dependent increases in oxidative damage to tissue proteins as evidenced by modification by lipid peroxides, radical adduction, and the formation of protein carbonyls (Pal et al., 2009). Increased DNA oxidation, detected as the formation of 8-hydroxy-2-deoxyguanosine (8-OHdG), was also a consequence of CEES exposure. Subsequent studies with the JB6 mouse keratinocyte cell line and primary fibroblasts in culture confirmed the ability of CEES to increase 8-OHdG and also increase both mitochondrial and cytosolic superoxide generation (Inturi et al., 2011). A report using mouse PAM212 keratinocytes in three-dimensional air−liquid interface cultures demonstrated concentration-dependent increases in hydrogen peroxide and protein carbonyl formation after topical application of CEES (Black et al., 2010a). These changes were accompanied by increases in the relative mRNA of Cu, Zn-SOD, catalase, thioredoxin reductase, and several glutathione-*S*-transferases (GSTs), suggesting a compensatory cellular response to oxidative stress.

There are likewise demonstrations that an increase in the antioxidant cellular capabilities of in vitro skin models reduces susceptibility to mustard alkylating agents. Several reports show a protection from SM, HN2, or CEES toxicity by activators of the transcription factor, Nrf2 (Gross et al., 2006; Abel et al., 2011; Udasin et al., 2016), as well as suggest that iodine, an agent demonstrated to protect the skin from HN2, may act via this mechanism (Ben-Yehuda Greenwald et al., 2017). Nrf2 translocates to the nucleus under conditions of electrophilic or oxidative stress and binds to the antioxidant responsive element, leading to increased transcription of antioxidant/electrophilic response genes (Li and Kong, 2009). Important mechanisms that are enhanced include the synthesis and availability of GSH, GST activity, and cellular efflux transporters. Finally, a variant of the HaCaT human keratinocyte line with increased resistance to SM has recently been developed (Schmidt et al., 2016). These cells, which also show resistance to cancer chemotherapeutic agents of several classes and to H_2O_2, are distinguished by having significantly higher resting GSH levels as well as a more robust rebound in GSH levels after SM exposure (Rothmiller et al., 2018a). In summary, there is now compelling evidence that the loss of cellular reducing equivalents and the increase in oxidative stress are key contributors to cutaneous toxicity by SM and its vesicating analogs.

38.3.1.3 Reactions with glutathione/calcium homeostasis

Depletion of GSH also affects calcium homeostasis; treatment of primary human epidermal keratinocytes with buthionine sulfoxamine depressed the level of reduced glutathione but increased intracellular Ca^{2+} (Ray et al., 1993). Neuroblastoma cells treated in vitro with 0.3 mM

SM maintained high cell viability for nearly 9 h, which then decreased with time. The decrease in cell viability was prefaced by an increase in free intracellular calcium that occurred between 2 and 6 h postexposure (Ray et al., 1995). Intracellular free calcium is a well-recognized marker of cell stress (Ruff and Dillman, 2007). Induction of intracellular calcium is thought to contribute to apoptosis induced by SM exposure in keratinocytes in vitro (Rosenthal et al., 2003). Despite these findings, Sawyer's group has shown that the sensitivity of primary human keratinocytes to SM was unaffected when calcium was reduced using chelators or increased using the membrane ionophore ionomycin (Sawyer and Hamilton, 2000). These findings suggest that although calcium levels are significantly altered by SM treatment, modulation of calcium alone is neither necessary nor sufficient for SM-induced apoptosis.

38.3.2 Inflammation

Inflammation is one of the major driving forces of the skin pathology caused by SM exposure. The contribution of cytokines to the inflammatory events is well established and their signaling pathways are important targets of medical intervention for potential countermeasures of vesicant injury (Joseph et al., 2016; Wagner et al., 2019). The literature linking inflammation and wounding is so extensive as to fill a book by itself. This section will therefore only present an overview of the inflammatory response to dermal skin injury. In short, cytokines signal via several pathways, including Akt, ras, Death Domain, MAP/Erk, and Janus kinase (Jak) (O'Shea and Murray, 2008; Weber et al., 2010; Kalliolias and Ivashkiv, 2016). The latter, JAK-signal transducer and activator of transcription (Stat) pathway, is a major regulator of all the cell types involved in inflammation and they can be either positive or negative mediators to the inflammatory process. Both proinflammatory and antiinflammatory events are under the influence of the Jak-Stat pathways and hundreds of cytokine receptors are regulated via these pathways, including more than 40 type I and type II cytokine receptors, and receptors for interleukins, interferons, growth stimulating factors, leptin, erythropoietin, and many more (O'Shea and Murray, 2008).

As for vesicant-injured skin, the inflammatory response appears to be biphasic. This is based upon our years of experience in addition to others who reported that inflammation played a minor role in the initial events of SM-induced cutaneous injury, but had much greater importance at later stages (Papirmeister et al., 1991b). Indeed, in another examination, the authors argued that inflammation may be more significantly involved in the vesication (blistering) event than previously believed, with inflammatory cells and mediators contributing directly to the formation of the primary lesion (Cowan and Broomfield, 1993). More recently, Joseph et al. (2011) documented a correlation between markers of inflammation and DNA damage with structural changes in skin following SM exposure.

Numerous accounts, from both in vivo and in vitro studies, have now documented a rise in a number of inflammatory cytokines in response to SM. Applications of SM in the MEVM have resulted in induction of IL-1β, IL-6, TNFα, and GM-CSF within 6 h (Sabourin et al., 2000; Wormser et al., 2005; Chang et al., 2018b). While IL-1α was not detected in these studies, its inducibility by SM vapor was confirmed in experiments in which the backs of hairless mice were exposed (Ricketts et al., 2000). Further, relative mRNA levels of IL-1β, IL-6, IL-8, and TNFα have been demonstrated to significantly rise within 24 h of SM vapor treatment in full-thickness skin biopsies from weanling pigs (WPs; Sabourin et al., 2002). Studies with cultured human keratinocytes have shown that these cells respond directly to SM with the production of cytokines. Interleukins-1β, -6, -8, and TNFα were detectable in culture media from these cells 24 h after treatment with 100−300 μM SM (Arroyo et al., 2000). Cultured skin fibroblasts have also been shown to express TNFα in response to SM. The chemotactic activity of keratinocyte-derived IL-8 could initiate the transmigration of circulating granulocytes into SM-exposed tissues where they could contribute to the primary lesion. Other cytokines may be involved in priming and activation of the recruited immune cells. While cytokine induction may occur via several means, there is now ample evidence that the inflammatory transcription factor NF-κB is one such pathway activated by SM (Atkins et al., 2000; Minsavage and Dillman, 2007; Rebholz et al., 2008). In fact, enhanced synthesis of the aforementioned cytokines in resident skin cells may occur as a result of this NF-κB activation (Ghosh et al., 1998). More recently, evidence has been provided that the p38/MAP kinase pathway is also important in SM-induced cytokine production by human keratinocytes (Ruff and Dillman, 2010).

Additional inflammatory mediators have been detected in cutaneous tissues as a consequence of SM injury. These include free arachidonic acid (Lefkowitz and Smith, 2002) and its cyclooxygenase (Rikimaru et al., 1991; Dachir et al., 2004) and lipoxygenase products (Tanaka et al., 1997). Furthermore, the increased capillary permeability observed would allow a variety of circulating inflammatory participants, such as complement components, kininogens, etc., to enter the dermal interstitium (Rikimaru et al., 1991). Clearly, vesicant injury involves a host of inflammatory mediators similar to those seen in other types of wounds, where individual cytokines have been singled out as potential therapeutic targets.

38.3.3 Protease activation

While there may be protease involvement in the initial SM injury, most studies have viewed the participation of serine and matrix metalloproteases (MMPs) in SM injury as a downstream event since MMPs in particular regulate various inflammatory and repair processes (Parks et al., 2004). The regulatory role confounds the potential use of MMP inhibitors as therapeutic agents because of their beneficial role in wound repair and remodeling. Increased protease activity following SM exposure has been reported in vitro in human peripheral blood lymphocytes (PBLs) (Cowan and Broomfield, 1993) and human epidermal keratinocytes (Smith et al., 1991), ex vivo in rabbit skin organ cultures (Higuchi et al., 1988; Woessner et al., 1990) and human skin explants (Rikimaru et al., 1991; Lindsay and Rice, 1996), in vivo in hairless guinea pig (HGP) skin (Cowan et al., 1993, 1994; Kam et al., 1997), and in vivo in the mouse ear (Powers et al., 2000) and hairless mouse (HM; Casillas et al., 2000a). The continued assessment of proteolytic activity in animal models is useful for characterizing specific proteases important to SM injury and for identifying effective protease inhibitors with therapeutic use in reducing or eliminating tissue injury caused by SM cutaneous exposure. The relationship between SM-increased protease activity and the subsequent vesication that occurs in SM lesions remains unclear, but emerging literature implicating protease involvement is consistent with the known ability of proteases to degrade basement membrane components in vitro and in vivo. Inhibition of these proteases should reduce the extent of the injury, promote a more rapid recovery, and provide a useful adjunct to other therapeutic strategies aimed at preventing SM-induced degenerative pathophysiological events (Cowan et al., 1993).

38.3.4 Apoptosis

Mechanisms underlying SM-induced apoptosis have been carefully explored using primary cultures of human keratinocytes. Treatment of keratinocytes with 100−300 μM SM resulted in activation of both caspase 8, which initiates the Fas-dependent death receptor pathway, and caspase 9, which initiates the mitochondrial apoptotic pathway (Rosenthal et al., 2003). Fas and Fas ligand (FasL) were upregulated in a concentration-dependent manner by SM leading to activation of caspase 3, the central executioner protease. Transfection of immortalized keratinocytes with a dominant negative Fas-activated death domain resulted in a blunted caspase response to SM. Microvesication and tissue injury produced in vivo by SM exposure of transfected cells after grafting onto athymic nude mice was also reduced by this treatment.

Changes in intracellular calcium levels are known to activate the mitochondrial pathway of apoptosis. A key regulator to Ca^{2+}-dependent proteins is calmodulin. SM has been shown to cause a time-dependent induction of calmodulin in keratinocytes (Simbulan-Rosenthal et al., 2006). Moreover, depletion of calmodulin using antisense probes attenuated SM-induced activation of caspases involved in the mitochondrial pathway of apoptosis. Both antisense and pharmacological inhibition of calmodulin prevented SM-induced nuclear fragmentation in the keratinocytes. Bad, a proapoptotic Bcl-2 family member present in an inactive phosphorylated form in viable cells, was also activated by SM. Furthermore, cyclosporine A, a selective inhibitor of calcineurin, a Bad phosphatase, inhibited SM-induced keratinocyte apoptosis. These results suggest that calcium-dependent activation of Bad may be a mechanism by which SM induces apoptosis in keratinocytes.

Another potential signal for SM-induced cell death is endoplasmic reticulum (ER) stress. An accumulation of unfolded proteins in the ER will trigger multiple corrective mechanisms, which, if unsuccessful, will lead to caspase-12-dependent apoptosis (Oyadomari and Mori, 2004). Using the MEVM, Chang et al. (2013) have identified specific molecular markers indicating induction of ER stress within 24 h of SM exposure.

One form of cellular demise common to epithelial cells is detachment-initiated apoptosis, also referred to as anoikis (Frisch and Francis, 1994). Epidermal keratinocytes rely on signals derived from the surrounding ECM for survival. It is possible that loss of these signals plays a role in SM-induced epidermal cell injury, and that cell detachment from the basal lamina precedes cytotoxicity. Several lines of evidence support this possibility. First, SM can alter the dynamics of cytosolic proteins that exert control over the attachment of cells to the basement membrane. For example, SM can modify intracellular actin microfilaments and keratin intermediate filaments known to be important in maintaining epithelial cell connections with the basal lamina. Thus, Hinshaw et al. (1999) reported that SM causes changes in the actin microfilament architecture and morphology of human keratinocytes within 3 h of exposure. This was associated with a significant decrease in keratinocyte adherence without evidence of cytotoxicity. In addition, Werrlein and Madren-Whalley (2000) found that SM caused rapid, significant decreases in immunodetection of keratins 5 and 14, an intermediate filament pair found in undifferentiated keratinocytes. In both in vivo (Gunhan et al., 2004) and in vitro studies with human keratinocytes (Dillman et al., 2003), and with purified proteins (Hess and FitzGerald, 2007), keratins 5 and 14 have been found to be alkylated by SM as well as nitrogen mustard (mechlorethamine, HN2) and CEES, the monofunctional analog of SM. Sites

of alkylation may be similar to dominantly acting mutations in keratins 5 and 14 that are known to be responsible for the human blistering disorder, EB simplex, in which, like SM-induced blistering, basal epidermal cells are targeted (Fuchs, 1997). The keratin cytoskeleton of basal keratinocytes links to the hemidesmosome and makes connections, through plectin, with the β4 cytoplasmic tail of integrin α6β4, thereby strengthening adhesion to the basement membrane via laminin-332 (Giancotti and Tarone, 2003). Alkylation of keratins 5 and 14 could cause aggregation and loss of function of the intermediate filament network and serve as a prelude to basal cell separation from the basement membrane. Likewise, as has been hypothesized, SM-induced proteolytic degradation of laminin-332 could provide the basis for blistering that occurs following SM exposure (Jin et al., 2016; Chang et al., 2018a).

In addition to its actions on epidermal cells, SM can directly alkylate ECM proteins in the skin, a process that can also alter the ability of basal keratinocytes to maintain vital connections with the basement membrane. In support of this idea, Gentilhomme et al. (1998) showed that SM treatment of human dermal equivalents reduced the ability of naïve keratinocytes to deposit laminin at the dermal−epidermal interface. In addition, Zhang et al., found that treatment of fibronectin and laminin with SM interfered with the ability of human epidermal keratinocytes to adhere to these matrix proteins (Zhang et al., 1995a,b). This inhibitory action was determined to be alkylation-dependent, because it could be prevented by cotreatment with SM scavengers. Sulfur mustard and nitrogen mustard also reduce cell and tissue immunoreactivity for laminin-332, as well as integrin α6β4 and collagen XVII (also known as bullous pemphigoid antigen), two hemidesmosomal components that are critical for keratinocyte adherence (Smith et al., 1997c, 1998; Zhang and Monteiro-Riviere, 1997; Werrlein and Madren-Whalley, 2000; Kan et al., 2003). Interestingly, each of these proteins, like keratins, has been implicated in human blistering disorders involving separation of the epidermis at the dermal−epidermal junction (Pulkkinen and Uitto, 1998; Yancey, 2005; Chang et al., 2018a). These findings suggest that SM can alter the interaction of basal cells with matrix proteins critical for basement membrane detachment. The alteration may occur by reducing protein functionality, decreasing protein expression, or increasing protein degradation. By whichever mechanism, a negative alteration in these critical anchoring components could lead to basal cell detachment and initiate anoikis.

38.3.5 Signal transduction pathways

Sulfur mustard exposure induces the activation of many molecular signaling pathways (reviewed in Ruff and Dillman, 2007). These pathways mediate many responses including inflammation, cell proliferation, cell differentiation, and apoptosis. Some that involve inflammation include the transcription factor NF-κB and p38 MAP kinase. NF-κB is a transcription factor that is induced within 2−4 h after SM exposure (Minsavage and Dillman, 2007). Dillman has suggested that this delayed induction is due to a nontraditional pathway of stimulation, whereby p90RSK phosphorylates IκB or the p65 subunit of NF-κB (Ruff and Dillman, 2007). The MAP kinase p38 is activated in response to damaging stimuli, including heat, UVA, and proinflammatory stimuli. Dillman's group and others have noted an increase in activation of p38 by phosphorylation and demonstrated that inhibition of p38 phosphorylation resulted in a decrease in SM-induced proinflammatory cytokine production in vitro (Kehe et al., 2009). However, the importance of NF-κB or p38 activation in mediating SM cutaneous injury has not been demonstrated in vivo. Also, while important for understanding SM-mediated toxicity, these pathways are difficult to target pharmacologically.

38.4 Models of dermal sulfur mustard exposure

38.4.1 Introduction

Although there has been considerable investigation of SM therapies since the publication of Bruno Papirmeister's landmark compilation of *Medical Defense Against Mustard Gas* nearly 30 years ago, no suitable pharmacological treatment for SM exposure to the skin has been approved (Papirmeister et al., 1991c). The field has dramatically changed direction from strategies and therapies based on protection from SM exposure to therapies designed to subvert the blistering process and increase the rate of wound healing. New pharmaceutical strategies will be increasingly focused on combination therapies such as bifunctional drugs that target multiple processes in blister formation and the wound-healing process, together with increased recognition of the importance of pharmaceutics.

Although many advances have been made in the production of skin barrier protection or postexposure skin decontaminants [such as skin exposure reduction paste against chemical warfare agents and reactive skin decontamination lotion (RSDL), respectively], drug countermeasures against vesicants remain a subject of intense investigation. The primary treatment strategy following exposure to vesicants such as mustard gas is decontamination. However, given the high reactivity of SM, there is a very short timeframe (3−5 min) in which decontamination can be effectively accomplished (reviewed in Vogt et al., 1984 and Wormser, 1991); and some studies

suggest that an extractable reservoir persists for much longer. Furthermore, because SM exposure does not cause immediate pain or noticeable effects, exposure is often not recognized until the effective window for decontamination has passed. Therefore, much research has focused on the identification of treatments that can be performed after exposure has occurred. These treatments can be directed at any stage during the progression of injury resulting from vesicant exposure, including before or after blister formation. This chapter focuses on the progress that has occurred in the 30 years since Papirmeister's publication (Papirmeister et al., 1991c).

38.4.2 Model systems for screening sulfur mustard

In vitro and in vivo models for SM have substantially improved over the past 5 years. Experimental animal models have different skin characteristics including a reduced barrier for chemical penetration as compared to human skin (Bartek et al., 1972). The reduced barrier results in greater systemic toxicity in animals, complicating other measures of cutaneous injury. Despite these difficulties, animal models have displayed great utility, and surrogate endpoints such as microblister (epidermal−dermal separation) formation or edema are established biomarkers for measuring the efficacy of candidate compounds.

A systematic characterization of animal models including the euthymic HGP, WP, the MEVM, and the HM showed that SM-induced subepidermal blister formation and epidermal cell death in all models tested (Smith et al., 1997b). Hairless mice are useful models of human skin; the absence of hair on the skin and increased skin thickness reduce the rapid penetration of toxicants (Walter and DeQuoy, 1980). Thus, the HM has emerged as an effective model for characterizing vesicant injury mechanisms and for early screening of candidate therapeutics (Blank et al., 2000; Casillas et al., 2000a; Ricketts et al., 2000; Sabourin et al., 2003; Pal et al., 2009; Tewari-Singh et al., 2009, 2010, 2011, 2013, 2014a; Anumolu et al., 2011; Dorandeu et al., 2011; Jain et al., 2011; Joseph et al., 2011; Vallet et al., 2012).

First characterized in 1967, the HGP has been the most widely used model for vesicant exposure (Marlow et al., 1990a,b; Mershon et al., 1990; Yourick et al., 1991, 1992, 1993, 1995; Cowan et al., 1993, 1994; Graham et al., 1994; Smith et al., 1995;Kim et al., 1996; Kam et al., 1997; Millard et al., 1997; Dachir et al., 2010; Benson et al., 2011). Like human skin, SM-treated HGPs experience defects in epithelial cell regeneration, basement membrane modifications, and increased necrosis and apoptosis, making them a good model for therapeutic testing (Kan et al., 2003; Dachir et al., 2006). There is one report utilizing the rabbit ear as a model of SM injury, however, we know of no reports further developing this model to screen candidate antivesicants (Zlotogorski et al., 1997).

The MEVM is performed by application of vesicant to the inner (medial) side of the ear (Casillas et al., 1997). Edema can be quantified by measuring 8 mm diameter ear punch biopsy weights. Microscopic investigation shows clear histopathologic changes that include edema, epidermal−dermal separation, and necrosis. These effects occur in a dose-dependent manner. This model has been used to screen many compounds and remains the most cost-effective live animal screen available (Casillas et al., 2000b; Dachir et al., 2002, 2004; Babin et al., 2003; Casbohm et al., 2004; Sabourin et al., 2004; Wormser et al., 2004a,b, 2005; Kiser et al., 2005; Amitai et al., 2006; Dillman et al., 2006; Kumar et al., 2015; Liu et al., 2016; Achanta et al., 2018; Chang et al., 2018b; Tumu et al., 2018). Furthermore, the benefits of a fully sequenced mouse genome and wide range of antibodies available make this model superior. Systemic effects do occur, as physiological and toxicological effects are detected in the contralateral (untreated) ear of animals (Babin et al., 2000).

WPs and minipigs have also been used extensively for SM-induced skin injury (Lindsay and Rice, 1995; Brown and Rice, 1997; Graham et al., 1999, 2000; Chilcott et al., 2000; Logan et al., 2000) (Fig. 38.3) due to established similarities between human and pig skin (reviewed in Graham et al., 2005). Erythema was found to peak at 24 h after a 15-min vapor exposure, with maximal edema occurring at 48 h postexposure (Smith et al., 1996, 1997b; Graham et al., 1999). The dermal−epidermal junction was also damaged at 48 h, with laminin-5 (renamed laminin-332) showing a progressive decrease in protein expression following SM injury (Smith et al., 1997c). Furthermore, inflammatory markers including IL-8, IL-6, IL-1β, and MMP-9 are induced within 72 h of treatment with SM (Sabourin et al., 2002). The WP is also used as a model of wound debridement to enhance repair (Graham et al., 2002a,b, 2006; Reid et al., 2007; Dalton et al., 2008), as discussed in greater detail later in this chapter.

The isolated perfused porcine skin flap (IPPSF) model is an effective in vitro model for SM exposure, characterized extensively by Monteiro-Riviere's laboratory (King and Monteiro-Riviere, 1990). Skin flaps consist of isolated, perfused skin with intact dermis and epidermis maintained by microcirculation in a system that mimics normal blood flow. Unlike other animal models, gross blisters are obtained, with blister-induced epidermal−dermal separation occurring at the upper lamina lucida (Monteiro-Riviere and Inman, 1995, 1997; Riviere et al., 1995). This model has allowed for several

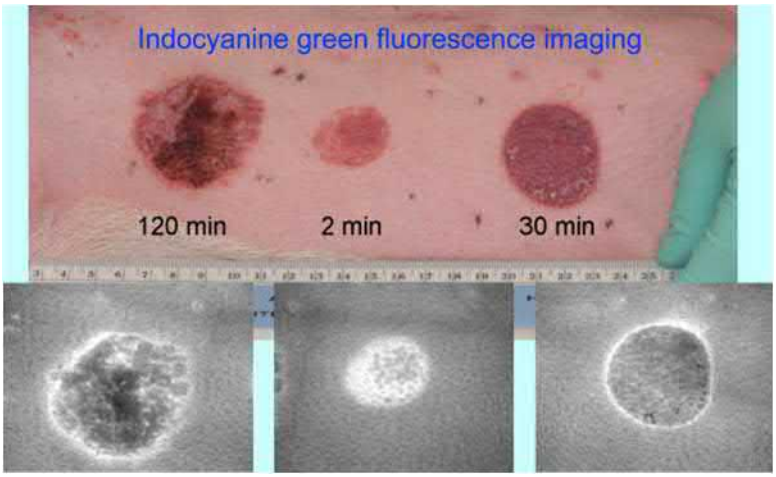

FIGURE 38.3 Weanling pig skin stained with indocyanine green following 48 h exposure to SM. *Reprinted from Graham, J.S., Chilcott, R.P., Rice, P., Milner, S.M., et al., 2005. Wound healing of cutaneous sulfur mustard injuries: strategies for the development of improved therapies. J. Burns Wounds 4, e1, in accordance with the Creative Commons Attribution License. (Graham et al., 2005)*

interesting experiments which could not have otherwise been performed. For example, the flow rate and composition of perfusing media can be altered: a higher flow rate is associated with increased blister formation, whereas increased glucose in the perfusion medium has been shown to block the formation of microvesicles and blisters (Riviere et al., 1997). Whether increased glucose can protect against blister development in live animals has not been tested. No reports using this model have been published since 2004.

Cell lines have been used to test vesicant toxicity and the efficacy of therapies. These studies are impacted by the differences in solubility of the vesicants, with CEES and SM being lipid soluble, and NM being water soluble, and all chemicals being quite reactive with water. Still, due to their ease of use, cell lines remain an important early screen for many toxic agents and there have been efforts to further develop as models for use with vesicants. HaCaT cells are immortal adult human skin cells that differentiate and proliferate easily in vitro. A cell line resistant to SM was developed in order to identify biological pathways that might offer protection against vesicants. The line was developed by serial culture in the presence of gradually increasing concentrations of SM over 40 months. This line, named HaCaT/SM, showed an 8.2-fold increase in LC^{90} (Schmidt et al., 2016). It showed smaller nuclei perimeter, higher clonogenicity, and altered gene expression. The cell line was found to be resistant to other alkylating agents and cytotoxic drugs (Schmidt et al., 2018b), and showed higher glutathione levels, survival against H_2O_2 treatment, and lower expression of nrf2 and its targets GCLC and GST (Rothmiller et al., 2018a). The microRNA MiR-181 was also induced and was thought to contribute to the resistance of this line (Rothmiller et al., 2018b).

HaCaT cells have also been tested in coculture with THP-1 immune cells in an effort to identify and characterize the immunological component of vesicant injury using a cell line system (Balszuweit et al., 2014). Treatment of HaCaT cells with SM in the presence of THP-1 cells increased necrosis, apoptosis, and inflammation in HaCaT cells. However, if the THP-1 cells were added following SM exposure, the increased signs of cellular injury were not evident, suggesting that the THP-1 cells were responding to SM treatment directly. Necrosulfonamide, an inhibitor of necroapoptosis, blocked the induction of proinflammatory cytokines when given to cocultures of HaCaT and THP-1 cells treated with SM, suggesting a role for immune cell death and release of these cytokines, which then impact HaCaT cells (Menacher et al., 2019). Diclofenac reduced necrosis, apoptosis, and inflammation in the cocultured cells treated with SM (Menacher et al., 2018), and the herbal remedy berberine provided similar protection (Lang et al., 2018). The efficacy of the antiinflammatory drugs in preventing SM injury suggests that this method may allow for screening of other drugs for SM injury that function via the same mechanism. Furthermore, removal or addition of the THP-1 cells allows for distinction of the molecular pathways affected.

In addition to cell lines, artificial human skin has been used to test the effects of SM (Petrali et al., 1993). Human skin equivalent, commercially available as EpiDerm, is a fully differentiated artificial human skin with both a dermis and an epidermis (Monteiro-Riviere et al., 1997). Full-thickness models (EpiDerm-FT) have been evaluated for their potential use in SM models as well, in particular focusing on the dermal−epidermal junction and basement membrane components (Hayden et al., 2009). Studies by our laboratories demonstrated CEES-mediated gene induction in EpiDerm-FT and demonstrated the usefulness of this model in histological evaluation following vesicant exposure (Black et al., 2010b, 2011). EpiDerm-FT might also be used as an in vitro

assay for screening of potential medical countermeasures against vesicants, such as was done with liposome-encapsulated glutathione (Paromov et al., 2011).

Administration of the vesicants themselves is also an area of consideration. Whereas SM and CEES are both lipid soluble, NM is water soluble. For lipid-soluble compounds, the chemicals may be placed directly on the skin, or a vapor cup model may be used in which a cup is placed over a portion of skin and the vapor pressure of the agent results in skin exposure over a fixed surface area. Different strategies must be used for NM as it is water soluble and has negligible vapor pressure. A cutaneous patch model has been developed for NM exposure in the skin. This model using a glass microfiber filter delivery system with a semiocclusive patch that allows for easier application of NM over defined areas of the skin (Composto et al., 2016).

38.4.3 Decontamination

Skin decontamination can be an effective means of reducing or preventing injury from SM exposure when conducted within minutes of exposure (Sidell et al., 1997; Chan et al., 2013). Most SM decontamination strategies focus on its removal with solvents or adsorption of SM to inert substances, since preventing skin penetration of sulfur mustard is the most important factor. Even simple techniques such as washing with soap and water are effective (Aasted et al., 1987; Rejaei et al., 2010; Weibrecht et al., 2012; Chan et al., 2013; Graham and Schoneboom, 2013). Washing with a variety of other substances has been suggested, including kerosene, oil, gasoline, surgical spirits, and neutral sodium hypochlorite (Sollmann, 1919a,b; Chiesman, 1944; Jelenko, 1974; Gold et al., 1994; Wormser et al., 2002). Fuller's earth, a binding agent, is a well-established and effective means for the decontamination of exposure to liquid sulfur mustard, however, it is difficult to contain and uncomfortable to apply. Fuller's earth is clay-rich soil commonly used to purify hydrophobic materials, including oils and greases (Chilcott et al., 2001), and functions by binding in a near-irreversible manner to SM, therefore, preventing its absorption, association, and reaction with skin components. While Fuller's earth is a common standard to which other decontamination agents are tested, modifications of this compound and other related clay-like materials may be more efficacious (Lyle et al., 1984, 1986, 1987; Joiner et al., 1987). Other items, including flour and talcum powder, were shown to be effective in reducing the progression of injury (Van Hooidonk et al., 1983). However, decontamination is ineffective in cases of vapor exposures to SM, which occur over a longer time period (McNamara, 1960). These results suggest that nearly anything that could adsorb a hydrophobic product would be effective in reducing SM injury.

Another strategy for decontamination is active chemical neutralization, whereby the SM undergoes a chemical reaction to prevent its reaction with outside components. There have already been successes with this type of approach, with the best example being RSDL, a product approved for military use by the United States and a number of other nations (FDA, 2003), and recommended for mass civilian exposure (Mengs et al., 2012). RSDL has been demonstrated to be as effective as Fuller's earth in its decontamination properties (Taysse et al., 2007, 2011). The first generation of this product was composed of a solution of potassium 2,3-butanedione monooximate and its free oxime, diacetyl monooxime, in a mixture of monomethyl esters of polyethylene glycol. The solvent portion of the lotion is designed to solubilize chemical weapons away from the skin, while the oxime component readily reacts with mustards and with nerve agents, producing less toxic products (Sawyer et al., 1991a,b; Sun et al., 2015). In domestic pigs, treatment with RSDL 5 min after exposure to SM resulted in significantly less injury observed 3 days postexposure (Taysse et al., 2007).

Other approaches to chemical neutralization include the use of sodium thiosulfate as a reducing agent to function by reacting with activated SM (Hatiboglu, 1960; Owens and Hatiboglu, 1961; Bonadonna and Karnofsky, 1965; reviewed in McKinley et al., 1982). Original studies (WW1-1970) focused on its use as an injectable drug to prevent the bone marrow suppression seen with exposure to SM and related mustard agents. Systemic administration of this and other antioxidant molecules such as *N*-acetyl cysteine is discussed later in this chapter. Another thiol, 2,3-dimercapto-propane sulfonic acid, showed protection against injury in mice exposed to SM vapor (Pant et al., 2000).

Decontamination can also be accomplished by the application of creams containing fluorinated cross-linker monomers (Liu et al., 1999). Deactivation occurs when substances within the cream actively react with and decontaminate the agents. Care should be taken with administration of these creams, since they can trap chemical agents on the skin and prevent natural off-gassing. This may result in enhanced injury if inadvertently applied after exposure to a chemical agent. In some cases, perfluorinated creams have produced an 18-fold reduction in the rate of skin absorption (Chilcott et al., 2002).

Once dermal absorption of SM has occurred, it is generally accepted that SM is irreversibly bound to skin constituents, and therefore cannot be removed from the skin through the methods discussed above. However, some investigators suggest that there remains some fraction of unreacted SM in the skin, in what is often described as a skin reservoir. This reservoir is thought to contribute to ongoing injury by continually releasing SM, allowing

further damage to occur. Indeed, recent studies demonstrated that application of ^{14}C-labeled SM was extractable from an in vitro pig skin model for up to 6 h, however, the extract was not further evaluated to show skin toxicity (Dalton et al., 2004; Hattersley et al., 2008). Future work might focus on targeting this reservoir to prevent ongoing damage; no therapies are currently available for this purpose.

38.4.4 Treatment of blisters

The prolonged pathology observed in SM injury suggests two pathological mechanisms: an ongoing toxicity that contributes to a step-wise progression culminating in blister formation, and a healing process that has been subverted. This is quite unlike the wound healing of thermal injuries. Aspiration (removal of the fluid within the blister) and deroofing (removal of the epidermal layer that constitutes the roof of the blister) are the main courses of action taken for larger, coalesced blisters, in order to promote the healing process (reviewed in Jenner and Graham, 2013).

Physical debridement of tissues, the surgical removal of tissue beyond the epidermal (roof) layer, enhances the rate of wound healing (Graham et al., 2005) (Table 38.1). The slow rate of healing suggests that SM-modified proteins or other cellular components are preventing the normal healing process (Eldad et al., 1998). SM-mediated cross-linking of structural proteins such as laminins may not be easily repaired and may contribute to a delayed wound-healing response (Zhang et al., 1995a). This could occur by promoting prolonged inflammation or by preventing the normal wound closure event that occurs as dermal cells migrate across and repair the blister area. Early studies demonstrated that SM-induced lesions in the skin of WPs or Yucatan miniature pigs underwent faster wound healing when treating lesions by debridement after exposure to SM, whether the debridement was performed by CO_2 laser or surgically (Smith et al., 1997a,b,c; Rice et al., 2000; Graham et al., 2002a). Furthermore, when combined with skin grafting, debridement promotes wound repair (Graham et al., 2002b; Rice, 2003; Evison et al., 2006; Dalton et al., 2008).

A 2018 report of an accidental human exposure to SM in the laboratory was effectively treated with debridement (Schmidt et al., 2018a). In this case, a laboratory worker synthesized SM for use in creating a new type of plastics. After accidentally spilling it on his lab coat, the technician removed his coat but was unaware that the underlying clothing was also contaminated until 30 min after exposure. His abdomen and left forearm were exposed, with the abdomen forming a 2-cm red patch. Two hours after exposure, the lesions were treated with H_2O_2 and cortisone. Five hours postexposure, the patient presented with pronounced reddening of his abdomen, with blisters forming at 9 h. The patient presented at the Center for Severe Burn Injuries in Hamburg, Germany, for treatment. At this point and beyond, photographs were taken of the injury and its healing process (Fig. 38.4). Over the course of 12 days, the abdominal lesion progressed as a second-degree burn without further healing. The wound was excised and skin grafting was done 14 days postexposure using skin from the patient's right thigh. The wound healed without complications (Fig. 38.4). The wound was not completely debrided, and the region that was grafted with skin has a different coloration than the region that healed on its own (Fig. 38.4, +1-year panel).

Interestingly, the patient returned to the hospital 14 months after the incident due to his concern that SM might lead to carcinogenesis. He requested complete removal of the affected region and a second skin graft. The surgery was performed, and the wound healed with less scarring and noticeable difference in skin color (Fig. 38.5, +1-year panel). Physicians performed skin biopsies prior to the surgery to evaluate whether malignancy had occurred. The excised tissue was free of inflammation and showed no signs of malignancy. Skin biopsies showed normal dermis and epidermis structure. DNA hypermethylation was observed in the regions that were exposed to SM, however. Four years after the accident, which was 22 months after the second surgery, the physicians noted that the scars were mature, stable, and freely moveable. This case provides an example of two treatment approaches in the same individual.

38.5 Therapeutics

38.5.1 Antioxidants

Chemical scavengers may be used to inactivate free radical forms of SM or the oxygen or nitrogen radicals thought to be formed as a consequence of SM activation. If given early enough after SM exposure, such scavengers might directly deactivate SM and perhaps reduce the reservoir of SM present in the skin. However, scavengers seem to have efficacy beyond the lifetime of SM itself in the skin, suggesting that other physiological mechanisms such as inflammation and oxidative stress might be reduced by these agents (reviewed in Papirmeister et al., 1991c). Scavengers have mostly been tested for their ability to reduce leukopenia and death due to the systemic effects of mustard exposure, rather than the reduction of skin injury (reviewed in Papirmeister et al., 1991c). Although most work has focused on scavengers as therapeutics in pulmonary exposures where ongoing oxidative stress contributes to toxic outcomes such as pulmonary fibrosis or chronic obstructive pulmonary disease, there has been limited research on their efficacy in treating cutaneous injury.

TABLE 38.1 Comparison of dermabrasion (debridement) strategies for enhancing the rate of SM-induced skin injury repair.

Therapy	Time of administration	Agent route	Species/ system	Efficacy	References
Reactive skin decontamination lotion	Pretreatment	Topical	In vitro, human skin	18-fold reduction in skin absorption rate, may partially extract SM from the skin reservoir	Chilcott et al. (2002)
Dermabrasion	96 h post	Vapor, skin	Yucatan pigs (miniature, larger white)	Accelerated (≤3 ×) wound healing	Rice et al. (2000)
Debridement, CO_2 laser	6, 24, or 48 h post	Vapor, skin, 15 min	WP	Threefold fewer wounds. Improved histological skin structure	Graham et al. (1997)
Debridement, CO_2 laser	48 h post	Topical, liquid, 2 h	WP	Improved histological skin structure	Graham et al. (2000)
Debridement, xeroform petrolatum	48 h post	Topical, liquid, 2 h	WP	Skin elasticity similar to sham (non-HD-treated) pig skin	Graham et al. (2006)
Debridement, Scarlet Red Ointment	48 h post	Topical, liquid, 2 h	WP	Skin elasticity similar to sham (non-HD-treated) pig skin	Graham et al. (2006)
Debridement, surgical tangential excision	48 h post	Topical, liquid, 2 h	WP	Mild improvement in healing	Graham et al. (2000)
Debridement, surgical tangential excision and skin grafting	48 h post	Topical, liquid, 2 h	WP	Improved histological skin structure	Graham et al. (2000)
Debridement, Versajet	48, 72, and 96 h post	Not specified	WP	No improvement at day 14	Dalton et al. (2008)
Debridement, Compound W	48, 72, and 96 h post	Not specified	WP	No improvement at day 14	Dalton et al. (2008)
Debridement, Collagenase Santyl	48, 72, and 96 h post	Not specified	WP	No improvement at day 14	Dalton et al. (2008)

WP, Weanling pig.

Sodium thiosulfate, a potent antioxidant and scavenger, has been shown to be effective in reducing leukopenia and platelet depression when given systemically in treatment for nitrogen mustard, particularly when given prior to exposure (Owens and Hatiboglu, 1961; Bonadonna and Karnofsky, 1965; McKinley et al., 1982). In the IPPSF model, perfusion with sodium thiosulfate modestly reduced microvesicle formation and attenuated the vascular response (Zhang et al., 1995b). However, it has limited efficacy against skin injury (Vojvodic et al., 1985; Zhang et al., 1995a,b).

Glutathione depletion has been shown to occur in several tissues and cell lines in response to mustard treatment (Omaye et al., 1991; Ray et al., 1995; Kulkarni et al., 2006). Depletion of glutathione by buthionine sulfoximine treatment of isolated human leukocytes increased their sensitivity to SM toxicity (Gross et al., 1993). Given the critical role of glutathione in maintaining the intracellular reducing state of the cell, restoration or pretreatment with glutathione may protect against SM toxicity. Indeed, pretreatment of the basal epidermal keratinocyte cell line SVK14, the upper respiratory tract cell line BEAS-2B, or the lower airway type II epithelial cell line A549 with glutathione was shown to provide resistance to SM toxicity (Smith et al., 1997a; Andrew and Lindsay, 1998; Lindsay and Hambrook, 1998). Similar results were seen with other human cell lines, including G361, SVK14, HaCaT, and NCTC human skin cells (Simpson and Lindsay, 2005). Also, stimulation of glutathione concentration by pretreatment with glutathione itself or the cysteine precursor 10 mM L-oxothiazolidine-4-carboxylate was shown to be protective against SM in vitro (Gross et al., 1993; Amir et al., 1998). However, these early successes did not translate effectively to animal model

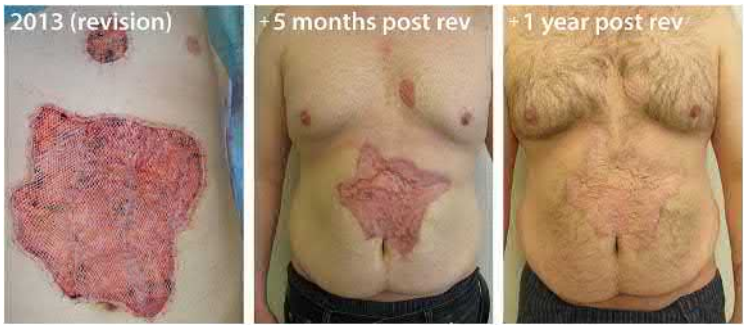

FIGURE 38.4 Primary healing phase of the upper body of a 27-year-old male laboratory worker exposed to liquid sulfur mustard over a period of 1 year. The injury was excised and split-skin grafting was performed 14 days after the exposure, and the patient returned to work on day 22 following the injury (Schmidt et al., 2018a). *Reprinted from Schmidt, A., Steinritz, D., Rudolf, K.D., et al., 2018a. Accidental sulfur mustard exposure: a case report. Toxicol. Lett. 293, 62–66, with permission from Elsevier.*

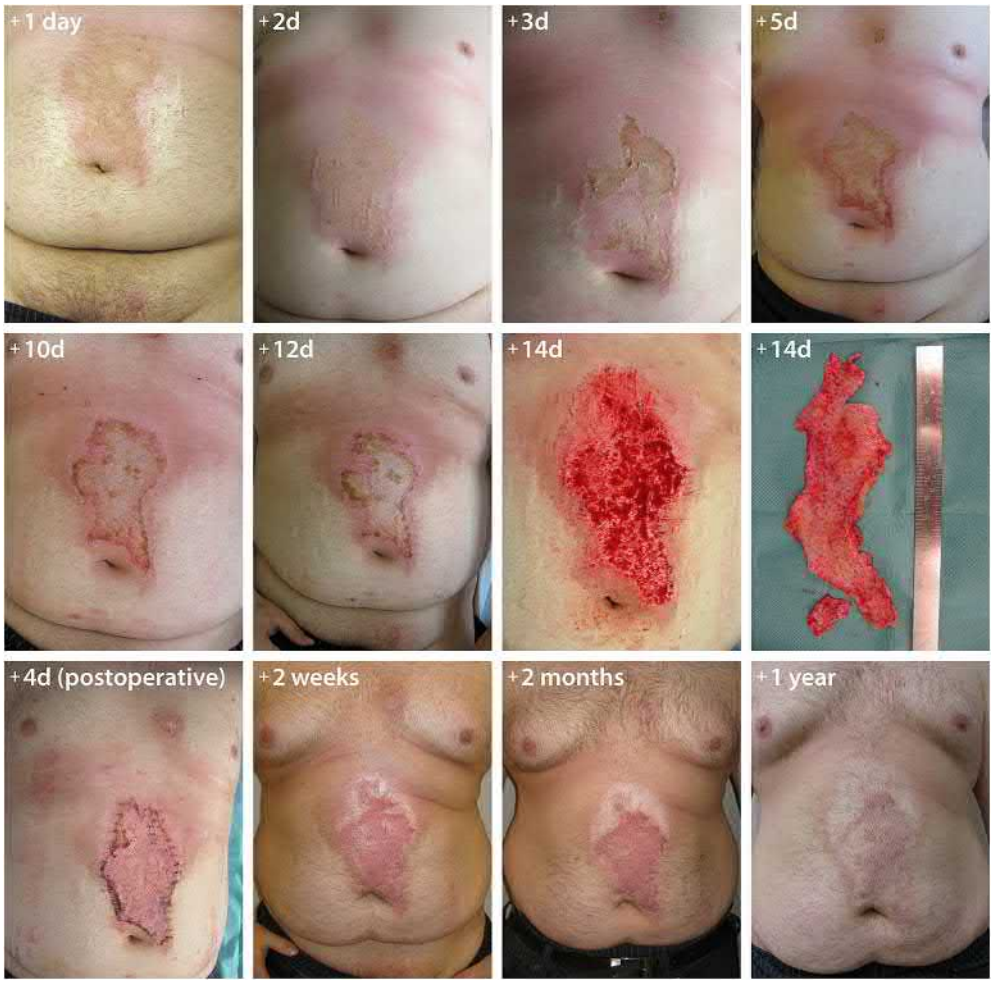

FIGURE 38.5 At approximately 1-year posthealing, the patient described in the previous figure was concerned about the possibility of cancer development, and requested the removal of skin tissue exposed to the SM. The affected areas were excised and covered with split-skin grafts taken from the right thigh (Schmidt et al., 2018a). *Reprinted from Schmidt, A., Steinritz, D., Rudolf, K.D., et al., 2018a. Accidental sulfur mustard exposure: a case report. Toxicol. Lett. 293, 62–66, with permission from Elsevier.*

systems. Reduced glutathione (400 mg/kg) given once before and twice after SM did not protect mice from SM toxicity (Kumar et al., 2001). Continued in vitro efforts have shown the protective effect of glutathione and glutathione-replenishment studies using in vitro models (Balsuweit et al., 2016). Because glutathione would be difficult to administer cutaneously, it is an unlikely therapeutic agent for the skin.

Cysteine is an amino acid with a reduced sulfur group that acts as an antioxidant. Cysteine residues are selectively alkylated in proteins by SM and the bifunctional nature of SM allows chemical cross-linking (Byrne et al., 1996). Early studies with nitrogen mustard in mice demonstrated that pretreatment with cysteine protected against toxicity (Contractor, 1963). However, microvesicle formation and dark basal cell formation were not protected in the IPPSF model (Zhang et al., 1995b). *N*-Acetyl-L-cysteine (NAC) acts both as a scavenger and as an inducer of glutathione synthesis, restoring the normal reducing status of the cell. Because SM lowers intracellular-reduced glutathione, its restoration may contribute to increased tissue survival and repair. In addition, pretreatment with NAC may elevate glutathione levels above normal and offer protection against low concentrations of SM (Atkins et al., 2000). In vitro, pretreatment with NAC protected PBLs from 10 μM SM (Gross et al., 1993). Endothelial cells pretreated with NAC were resistant to loss of cell adherence and rounding following exposure to 250 μM SM (Dabrowska et al., 1996). Liposomes containing NAC increased the viability of HaCaT keratinocytes in an in vitro study if given simultaneously with CEES (Paromov et al., 2008). Although antioxidants have proven to be efficacious if given prior to SM exposure, their ability to ameliorate skin damage or enhance wound repair has not been shown.

Silibinin is the main active flavonone of silybin, an extract of the milk thistle plant (*Silybum marianum*). Silibinin or silybin has been tested in clinical trials as a chemotherapeutic agent (Singh and Agarwal, 2005; Deep and Agarwal, 2010) for hepatitis, diabetic nephropathy, and other indications. The compound has diverse effects, including antiinflammatory, antioxidant, and antimetastatic effects (Gu et al., 2007; Singh and Agarwal, 2009; Deep and Agarwal, 2010). Topical silibinin (1 mg) attenuated CEES-mediated pathology in the skin of SKH-1 mice when applied 30 min post-CEES exposure, including epidermal thickness, apoptosis, induction of proinflammatory genes, lipid peroxidation, and DNA oxidation (Tewari-Singh et al., 2012). It also attenuated NM-mediated injury at 24, 72, and 120 h postexposure as measured by epidermal thickness, dead and denuded epidermis, parakeratosis, and microvesication, and reversed DNA damage as measured by 8-oxodG (Jain et al., 2015).

Ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] is an organo-selenium compound that functions as an antioxidant; it can directly scavenge hydrogen peroxide and peroxynitrite, mimics glutathione peroxidase, and acts as an antiinflammatory agent (Schewe, 1995). Ebselen itself reduced HN2-mediated inflammation at 24 h in the MEVM when applied in three treatments postexposure, with the first starting at 15 min. Several ebselen derivatives have shown efficacy in in vitro studies in skin and lung cell lines (Pino et al., 2013, 2014). In the MEVM, ebselen reduced HN2-induced tissue swelling (Lulla et al., 2014). An analog of ebselen, ebselen oxide (EB-2), reduced HN2-induced increases in wet weight, ear thickness, hyperplasia, vesication, inflammatory cell infiltration, apoptosis, and MMP-9 (Tumu et al., 2018).

AEOL-10150 is a broad-spectrum antioxidant that has been tested as an inhibitor for several chemical agents, including sulfur mustard (Zhang et al., 2018). In the HaCaT epidermal cell line model, AEOL-10150 reversed CEES-induced decreases in DNA synthesis and cell viability (Tewari-Singh et al., 2014a). It also improved skin bifold and epidermal thickness, myeloperoxidase activity, and DNA oxidation in mouse skin (Tewari-Singh et al., 2014b).

38.5.2 Poly(ADP-ribose) polymerase inhibitors

Although PARP inhibitors were one of the first sets of drugs to be investigated for use against vesicant injury, earlier studies did not yield positive results in animal model systems and this line of research was abandoned for many years (Casillas et al., 2000b). Recently, the development of PARP inhibitors with better pharmacologic properties for the treatment of cancers has led to a renewed interest in the potential for PARP inhibitors to treat SM injury. ABT-888, with efficacy against both PARP-1 and PARP-2, was approved as an orphan drug in 2016 by the FDA. In the HaCaT keratinocyte model, ABT-888 protected against CEES and SM-induced cell death and blocked vesicant-mediated depletion of NAD^+/ATP (Debiak et al., 2016; Mangerich et al., 2016). In the MEVM using Kunming mice, ABT-888 reduced edema and epidermal necrosis due to SM when given 30 min prior to SM exposure (Liu et al., 2016). A lingering concern is whether halting apoptosis of damaged cells can lead to carcinogenesis in the future.

38.5.3 Proteolytic inhibitors

Proteases play a critical role in wound repair and remodeling, and therefore are likely important targets for the enhancement of wound repair (Mohan et al., 2002). In particular, damage to the basement membrane by MMPs is thought to be one mechanism responsible for

TABLE 38.2 Summary of the effects of protease inhibitors on SM toxicity.

Therapy	Time of administration	Therapy route	Therapy concentration	Agent route	Species/ system	Efficacy	References
Doxycycline	Cotreatment, then posttreatment	In media	500 μM	In media	HaCaT cells, in vitro	Reduced cellular detachment, but did not prevent apoptosis	Lindsay et al. (2007)
Doxycycline	1 h post	In media	100 μM	In media, 200 μM	HEK keratinocytes, in vitro	Reduction of SM-induced IL-8 production	Nicholson et al. (2004)
Doxycycline	Cotreatment	Topical	90 μM	Topical	In vitro human skin explants	No effect on SM-induced dermal–epidermal separation	Schultz et al. (2004)
GM 1489[a]	Pretreatment 15 min	Topical	20 μL of 25 mM	Liquid	Mouse	Reduced expression of MMP-9 mRNA	Gerecke et al. (2005)
Ilomastat	Pretreatment 15 min	Topical	20 μL of 25 mM	Liquid	Mouse	No effect	Gerecke et al. (2004)
Povidone iodine	15 m and 24 h post	Topical	40 mg 10% povidone iodine	HN2, 0.5 mg	Haired guinea pig	>80% reduction in MMP-2 and MMP-9 activity in skin	Wormser et al. (2002)
Iodine	20 m post	Topical	1% w/v	Liquid, 1.27 mg	Pig	No effect	Margulis et al. (2007)

[a]*GM 1489, N-[(2R)-2,4-methylpentanoyl]-L-tryptophan-(S)-methyl-benzylamide.*

dermal–epidermal separation seen in SM-induced blister formation. This could occur by upregulation of MMP expression, reduced competition for MMPs by adhesion molecules, or both (Mol, 1999; Danne et al., 2001; Shakarjian et al., 2006). MMP-9 expression is upregulated in WP skin (Sabourin et al., 2002) and the MEVM following SM exposure (Chang et al., 2006). Inhibition of proteases might therefore ameliorate damage caused by SM to structural components of the skin (Table 38.2). Explant cultures of SM-treated human skin cotreated with Ilomastat showed no epidermal–dermal separation (Schultz et al., 2004). Sulfur mustard-induced MMP-9 mRNA in the MEVM was partially inhibited by pretreatment with the MMP-9 inhibitor GM 1489 (Gerecke et al., 2005). The antibiotic doxycycline is also an MMP inhibitor that was shown to attenuate SM-induced pulmonary and ocular injury (Guignabert et al., 2005; Horwitz et al., 2014). However, doxycycline does not protect HaCaT viability; cells lose adherence and undergo apoptosis (Lindsay et al., 2007).

Microvesication of human skin in vitro was inhibited by treatment with MMP inhibitors such as Illomastat, but HGP skin had no decrease in microvesication or necrosis following repeated treatments with Ilomastat (Mol and van den Berg, 2006). Mol and Van den Berg suggest that this may be due to lack of delivery of the MMP inhibitor to the site of action. Although MMP inhibitors may reduce the ongoing injury caused by upregulation of MMP-9, most inhibitors have difficulty penetrating the skin. Another study demonstrated that treatment of an ex vivo human skin model with the metalloprotease inhibitor GM6001 blocked microvesication even when given 8 h following HD exposure (Mol et al., 2009). The application of pharmaceutical concepts may aid in the development of better delivery systems that would enhance the efficacy of this class of drugs.

MMP-9 is released by fibroblasts in response to keratinocyte stimulation by sulfur mustard (Ries et al., 2008). Indeed, mustard-stimulated release of MMP-9 only occurred in coculture of human dermal fibroblasts with HaCaT keratinocytes or with culture of human dermal fibroblasts with conditioned media from HD-treated HaCaT cells, suggesting that paracrine signaling is responsible (Ries et al., 2008). Our work showed a dramatic increase in cytokine expression and activation of mediators of inflammation within 24 h after HD exposure, which may contribute to MMP-9 induction (Fig. 38.6) (Gerecke et al., 2009). Together these data suggest that inhibition of fibroblast activation following vesicant exposure may also reduce MMP-9 activation and release.

38.5.4 Steroids, corticosteroids, and glucocorticoids

Steroids have shown some efficacy in reducing inflammation and blister formation in response to SM or genetic blistering diseases such as bullous pemphigoid

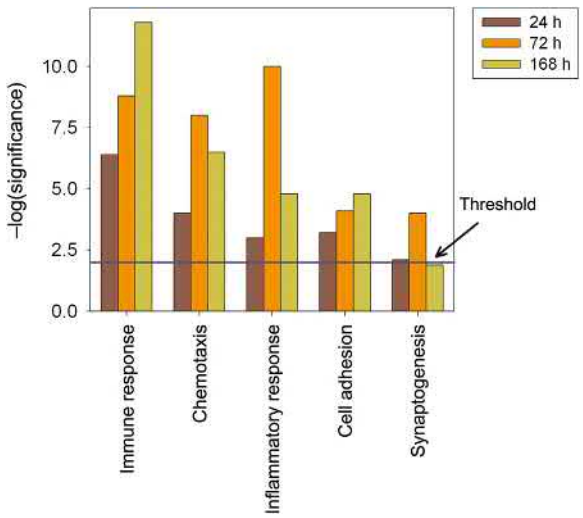

FIGURE 38.6 Results of biological processes sorting for microarray data from three different timepoints after sulfur mustard exposure using the mouse ear vesicant model. The timepoints were 24, 72, and 168 h postexposure. The bars represent positive fold change increases over the carrier solvent alone samples. The immune response genes were within the top three biological processes activated for all the timepoints studied (Gerecke et al., 2009).

(Di Zenzo et al., 2007). Early studies showed that several types of corticosteroids were effective in reducing edema induced by SM (Dannenberg and Vogt, 1982). Glucocorticoids were shown to be effective in reducing edema in the initial phase of injury, but did not affect the overall rate of healing (Vogt et al., 1984). Hydrocortisone given systemically or topically 2 h prior to HD administration resulted in a reduction in ear inflammation in the MEVM (Babin et al., 2000; Casillas et al., 2000b). Steroids given after SM exposure also enhance wound healing in a Yorkshire pig model of SM injury (Reid et al., 2008). Dexamethasone in combination with the nonsteroidal antiinflammatory diclofenac produced more than a 60% reduction in edema in mouse ears treated with SM (Dachir et al., 2004). However, reduction of edema, while important, did not necessarily correlate with a reduction in the progression of injury as seen later in this work. Studies using newer steroids such as clobetasol showed improved healing, lower severity of basal cell necrosis, and less inflammation (Reid et al., 2008). Cotreatment of steroids with nonsteroidal antiinflammatory drugs (NSAIDs) shows greater promise: cotreatment with diclofenac and tacrolimus in the HGP exposed to SM vapor showed less erythema, reduced lesion area, and fewer lesions (Dachir et al., 2008). A similar combination of a steroid (Adexone) and NSAID (Voltaren) applied to mouse ears treated with SM led to reduced inflammation, less edema, reduced area of clinical damage, and reduced damage to epithelial cells (Dachir et al., 2004). Steroids have been used in humans exposed to SM, including a recent accidental exposure (Schmidt et al., 2018a: 62), but they do not appear to affect the progression of the wound or alter the rate of wound healing.

38.5.5 Nonsteroidal antiinflammatory drugs

Several studies have demonstrated that NSAIDs given systemically or topically offer protection against continued SM-mediated toxicity. This suggests that inflammation is a key component of SM-induced injury as it is with other cutaneous injuries, such as those induced by UVA (Yourick et al., 1995). These results suggest that inflammation is involved in the ongoing pathology of SM-induced injury, and that antiinflammatory drugs should be considered as part of a drug cocktail for treatment of SM injury. Early studies with indomethacin in the HGP model showed that oral pretreatment could attenuate erythema and cutaneous injury (Yourick et al., 1995). Partial protection by indomethacin against microvesicle formation was found in the IPPSF model (Zhang et al., 1995b). Significant reductions in SM-induced early edema were found when indomethacin was administered from 24 h prior to exposure to 24 h after exposure (Babin et al., 2000) or when given 20 min post-SM challenge (Kiser et al., 2001). Topical indomethacin given 2 h prior to SM exposure in the MEVM protected against early (24 h) but not late (72 h) edema (Casillas et al., 2000b).

Early studies using the MEVM showed that posttreatment with NSAIDs, particularly in combination with steroids, could diminish SM-induced inflammation at early time points, although later effects were not measured (Dachir et al., 2002). As mentioned above, Voltaren, an NSAID, given in combination with the steroid Adexone, reduced skin injury (Dachir et al., 2004).

38.5.6 Bifunctional compounds

Bifunctional compounds include two active agents tethered chemically to one another. A bifunctional compound containing NSAIDs (such as ibuprofen or diclofenac) tethered to pyridostigmine, an acetylcholinesterase inhibitor, via an 8- or 10-hydrocarbon chain spacer, was somewhat effective against SM dermal toxicity (Amitai et al., 2005). This combination was effective in the MEVM in reducing subepidermal blistering (Amitai et al., 2006), although nothing has been published on these compounds since 2006.

Individually, antiinflammatories and COX-2 inhibitors have been shown to protect against vesicants (Wagner et al., 2019). NDH-4338 (Fig. 38.7), a bifunctional compound consisting of the antiinflammatory molecule indomethacin coupled via an aromatic ester-carbonate linkage to the anticholinergic choline bioisotere 3,3-dimethyl-1-butanol, was tested against NM delivered by cutaneous

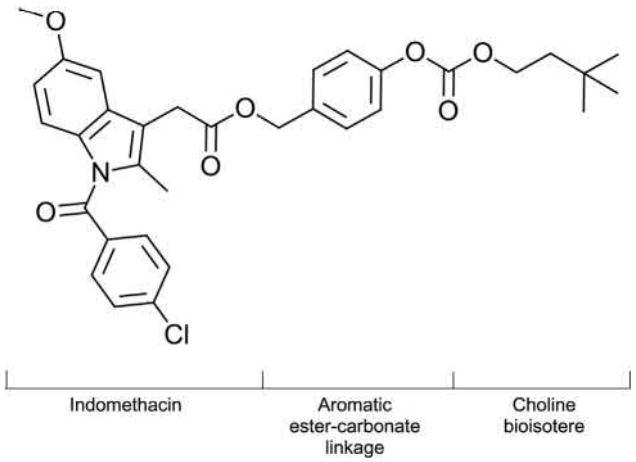

FIGURE 38.7 Chemical structure of the bifunctional anticholinergic NSAID compound NDH-4338. The drug combines two antiinflammatory moieties (indomethacin and a choline bioisotere, 3,3-dimethyl-1-butanol), attached via an aromatic ester-carbonate linkage (Chang et al., 2014). *Reprinted from Chang, Y.C., Wang, J.D., Hahn, R.A., et al., 2014. Therapeutic potential of a non-steroidal bifunctional anti-inflammatory and anti-cholinergic agent against skin injury induced by sulfur mustard. Toxicol. Appl. Pharmacol. 280 (2), 236–244, with permission from Elsevier.*

patch in the CD-1 mouse (Composto et al., 2016). NDH-4338 reduced wound thickness, eschar formation, mast cell degranulation, keratinocyte expression of iNOS and COX-2, and epidermal proliferation markers in CD-1 mice treated with NM. SKH-1 hairless mice treated with SM via a dorsal vapor cup showed that NDH-4338 protected against edema, enhanced reepithelization, reduced COX-2 expression, increased K10 expression in the suprabasal epidermis, reduced expression of K6, and restored basement membrane integrity through laminin-332 expression (Chang et al., 2014). A subsequent study investigating the role of mast cells using the vapor cup model showed that an ointment-based formulation of NDH-4338 reduced mast cell degranulation up to 14 days post-SM exposure (Joseph et al., 2018).

38.5.7 Transient receptor potential ligands

Transient receptor potential cation channels (TRPs) are ion channels that respond to environmental signals such as pain and heat. TRPs have been investigated as potential targets for SM toxicity for well over a decade. Nonmyelinated sensory C-fibers arising from the dorsal route ganglion transmit sensory information from the skin to the central nervous system in response to noxious stimuli, such as pain and heat. These fibers function as dual sensory efferents and release nociceptive and inflammatory neuropeptides such as substance P, peripherally (Szallasi and Blumberg, 1999; Szolcsanyi, 2004). Agents such as capsaicin, the active ingredient in hot peppers, produce analgesia by binding as an agonist to the transient receptor potential V1 channel (TRPV1) (Szolcsanyi, 2004). Capsaicin rapidly produces desensitization and interferes with the release of neuropeptides from sensory fibers (Campbell et al., 1993). Moreover, TRPV1 is expressed on cells in a number of nonneuronal cutaneous tissues, including keratinocytes and mast cells (Li et al., 2007). Capsaicin and its structural analogs, known collectively as vanilloids, have been shown to have antiinflammatory activity, as demonstrated by inhibition of edema, mast cell degranulation, and leukocyte migration (Brand et al., 1990; Bunker et al., 1991). Pretreatment of skin with vanilloids prior to SM exposure was shown to significantly reduce edema formation (Babin et al., 2000, 2003; Casillas et al., 2000b; Sabourin et al., 2003). One such vanilloid, olvanil, is a highly lipophilic analog of capsaicin, and has been shown to reduce SM-induced histological damage and edema as well as cytokine and chemokine mRNA induction (Casillas et al., 2000b; Sabourin et al., 2003). Analogs octyl homovanillamide and heptyl isovanillamide were shown to display similar protective activities against SM (Casbohm et al., 2004).

More recently, TRPA1 was shown to be activated by alkylating agents SM and CEES, inducing increased intracellular calcium; cells lacking TRPA1 did not respond with increased intracellular calcium (Stenger et al., 2015). Furthermore, AP18, an inhibitor of TRPA1, was found to block the effects of vesicants. Follow-on studies showed that TRPA1 antagonists (HC-030031 and A-967079) reduced CEES-induced skin edema, cytokines IL-1beta and CXCL1/KC, and MMP-9 in the MEVM (Achanta et al., 2018). Both TRPV1 and TRPA1 have been implicated in neurogenic inflammatory processes in mouse skin, suggesting that their inhibition might attenuate inflammation induced by vesicants (Horvath et al., 2015).

38.5.8 Cooling

Due to its high vapor pressure, exposure to SM in hot environments can exacerbate exposure. The toxicity of SM also relies on its ability to chemically react with biological molecules within the skin in a temperature-dependent manner. In vitro tests showed that human skin keratinocytes treated with SM and cultured at 25°C had less injury after 24 h than keratinocytes grown at 37°C (Sawyer and Risk, 1999). Similarly, HGPs with skin exposed to SM had less injury after 72 h if treated for 4.5 h postexposure with cold (Sawyer and Risk, 1999). Other studies showed that anesthetized swine skin exposed to mild cooling (15°C) for 2–4 h following SM exposure had significantly less injury progression after 7 days (Sawyer et al., 2002). However, later studies demonstrated that this effect was temporary, slowing the rate of injury progression rather than reducing overall injury. Indeed, tissue and animal studies showed that

temperature-mediated inhibition of injury was reversible upon return of the tissue to normal body temperature (37°C). Sawyer et al. (2002) have suggested that cooling might therefore be used as a temporary measure that "increases the therapeutic window in which other medical countermeasures are useful (Sawyer and Nelson, 2008)." Indeed, later tests with VX poisoning showed similar results, lengthening the time for entry of VX into the bloodstream (Sawyer et al., 2011). Although this work has been largely ignored by the literature, cooling would be inexpensive, effective, and easily applied by first responders.

38.6 Concluding remarks and future directions

Model systems for vesicants have reached a turning point, allowing elucidation of critical molecular pathways in vesicant injury that have allowed the development of therapeutics. NM and CEES have been validated as adequate surrogates for SM, and tremendous progress has been made in characterizing the most popular model systems for mustard, including the MEVM, SKH-1 HM, and WP models. The pharmacological targets remain largely the same as they were 15 years ago: inflammation, oxidative stress, and the TRP channels, with some renewed interest in the PARP and cholinergic pathways. Although early work showed limited success with drugs targeting these pathways, as the drugs in these categories have been refined for other purposes, their efficacy has improved in the vesicant model. Still, no therapeutic countermeasure exists that matches the efficacy of surgical intervention to remove damaged tissue exposed to vesicants, as was again shown with a critical human accidental exposure in Germany that was published in 2018 (Schmidt et al., 2018a: 62). This suggests that some molecular targets are critically and irreversibly damaged by vesicants and do not heal quickly on their own. Debridement and removal of the damaged tissues allows for healing of dermal wounds in a manner consistent with other injuries, rather than the prolonged healing process seen with SM.

Challenges remain for the development of pharmacologic countermeasures. Most of the research on pharmacological countermeasures for vesicants remains focused on the early time points of the healing process, but there is little evidence that targeting these early timepoints impacts the progression of the vesicant wound afterwards. The majority of studies covered in this chapter analyze the efficacy of countermeasures fairly soon after the injury occurred, and early in the healing process. Model systems that accurately model the late stages of vesicant wound healing remain to be characterized, presenting the largest problem going forward for countermeasure research. A second avenue for further research is combining pharmacological and surgical techniques to enhance the rate of wound healing. Finally, a greater focus on the interplay between inflammation and wound repair in vesicant injury is warranted.

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Chapter 39

Reproductive toxicity and endocrine disruption of potential chemical warfare agents

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39.1 Introduction

Since this chapter was originally written for the first edition of this book, the factual information about the effects of potential chemical warfare agents (CWAs) on reproduction has, for the most part, remained unchanged. However, where indicated, the results of recent research has been added for specific toxicants, such as sulfur mustard. Alarmingly, what *has* changed dramatically over the last decade is the ability of information to be communicated or miscommunicated globally. Internet availability, widespread cellular telephone access, and cybersecurity issues have all vastly increased the ability of misinformation, as well as disinformation (i.e., inaccurate information transmitted without malicious intent, versus communication of false information with malicious intentions, respectively), to be possibly manipulated and relayed to large populations of people. It is, therefore, easier than ever for disinformation to be intentionally weaponized, in order to create psychological and/or financial stresses in targeted populations (Forno, 2018).

Reproduction is a critical biological process in all living systems and is required for species survival. The immediate, short-term toxicological concerns regarding chemical warfare and acts of agricultural and industrial terrorism have to do primarily with human and animal incapacitation and mortality. Logically, most of the currently available literature on these aspects of warfare and terrorism focuses on these immediate adverse health effects and their sequelae. However, the entire scope of the effects of these toxic insults can involve the severe emotional and financial distress, as well as the diminished prognosis for long-term survival of exposed human and animal populations, arising from toxicant-induced abortions, congenital defects, and female and/or male infertility. With the advent of online information resources and social media, as well as lapses in cybersecurity, the ability to intentionally distribute nonfactual or misleading information, including that pertaining potential reproductive effects of potential CWAs, can be weaponized to sow fear and confusion in targeted human populations (Forno, 2018). It is therefore more important than ever before that individuals working in human and veterinary medicine, the military, public health, government regulatory agencies, industry and agriculture, as well as public policy makers, be familiar with some of the documented adverse reproductive effects of these types of toxicants on exposed humans and animals. This chapter will review what is currently understood about the potential adverse effects of chemical warfare agents, nuclear fallout, and hazardous industrial and agricultural wastes on human and animal reproductive function. In addition, the rapid intentional distribution of false information about potential CWAs can be viewed as an emerging technological threat.

For the purposes of this chapter, the term "reproduction" will be used primarily in reference to vertebrate species of animals (especially mammals) and will be inclusive of "development" (Fig. 39.1), which is sometimes treated as a separate topic in toxicology texts. This chapter will emphasize what is currently known about the adverse effects of known chemical warfare agents and selected environmental contaminants on male and female reproductive function, as well as xenobiotic-induced effects on the growth, maturation, and sexual differentiation of the embryo and fetus. Endocrine disruption is an

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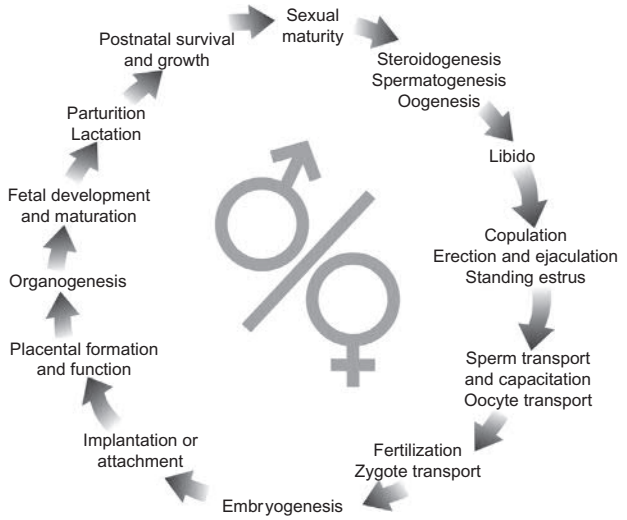

FIGURE 39.1 The multiple steps involved in reproductive development and function in both males and females are shown schematically to illustrate the complexity of reproduction in mammalian species and to demonstrate the various stages in the reproductive process which can be targeted for toxic insult. *Adapted, with permission, from Ellington, J.E., Wilker, C.E., 2006. In: Peterson, M.E., Talcott, P.A. (Eds.) Small Animal Toxicology, second ed. Elsevier Saunders, St. Louis (modifications and artwork courtesy of Don Connor).*

extremely common mechanism of action for xenobiotics associated with impaired reproductive function and will be discussed along with reproductive toxicity in this chapter. Efforts will be made to clarify the currently used terminology related to these topics and to provide the reader with a brief description of proposed mechanisms of action and observed reproductive outcomes associated with selected toxicants which might be relevant to chemical warfare and/or acts of terrorism.

Unfortunately, although there is a relative lack of information documenting long-term, reproductive effects of the types of toxicants covered in this book, space constraints still limit the amount of information which can be presented in this chapter. There are a number of recently published textbooks and book chapters which cover some of these and related subjects in greater detail and provide information which is complementary to what is presented in this chapter (Capen, 2008; Evans, 2017, 2018; Foster and Gray, 2013; Golub, 2006a,b; Gupta, 2006, 2018; Naz, 2005; Rogers, 2013; Romano et al., 2008; Senger, 2012). This is especially true with respect to normal reproductive anatomy and function (Evans, 2018; Evans and Ganjam, 2017; Senger, 2012), and the reader is directed to these publications and the other references cited in this chapter in order to gain additional insight into specific areas of reproductive function and toxicology.

It is important that the reader understand that the areas of toxicology involving the long-term effects of chemical warfare agents and environmental contaminants, reproductive toxicity, in general, and endocrine disruption, in particular, are in continual flux. New data and exceptions to "classical" mechanisms of action are being reported on a regular basis, and there continues to be ongoing debate about the effects of chronic low-level exposures to toxicants available for or arising from military and terrorist activities and various aspects of normal, as well as xenobiotic-induced abnormal reproductive function. Every effort has been made to accurately represent what is currently understood about the topics of discussion in this chapter. Controversial topics or those currently still subject to debate within the scientific community have been noted wherever possible.

39.2 Important definitions and concepts

39.2.1 Chemical warfare agents

For the purposes of this chapter, "chemical warfare agent" (CWA) will be used as a fairly comprehensive term to refer to a diverse group of toxicants commonly discussed within the context of chemical incapacitation for crowd control during riots or death or incapacitation associated with military use or terrorism. The lacrimatory and irritant riot control agents will include α-chlorbenzylidene malonitrile (CS), dibenz (b,f)-1:4 oxazepine (CR), ω-chloroacetophenone (CN), and oleoresin of capsicum (OC pepper spray) (Salem et al., 2008a). The CWAs currently of greatest interest and for which the greatest amount of data have been gathered are arsenicals, chlorine gas, phosgene and phosgene oxime, sulfur mustard, ricin, hydrogen cyanide and cyanide-related compounds, and organophosphate nerve agents (Kikilo et al., 2008; Wismer, 2018).

39.2.2 Environmental contaminants associated with industrial or agricultural terrorism

For the sake of completeness, this chapter will also discuss the adverse reproductive effects of potential toxicants, such as ionizing radiation, pesticides, and other organic environmental contaminants, as well as heavy metals, which are known to adversely affect reproductive function. While all of these potential toxicants have been or are currently being released into the environment in generally low concentrations as a consequence of normal industrial and agricultural activities, the results of accidents, such as those which occurred in Chernobyl, in the former Soviet Union (ionizing radiation), Bhopal, India (methyl isocyanate or MIC), Seveso, Italy and Times Beach, Missouri (dioxins), and Minamata, Japan and Basra, Iraq (methyl mercury), underscore the potential impact that large-scale, industrial or agricultural terrorism-related releases of these contaminants could have on populations of humans and animals. In fact, these

environmental contaminants have the potential to become "low-tech" CWAs in the hands of terrorists and less sophisticated military organizations.

39.2.3 Reproduction

As represented in Fig. 39.1, reproduction in humans and domestic, wild, and laboratory vertebrate animals encompasses the wide range of physiological processes and associated behaviors and anatomical structures involved in the production of the next generation and the survival of a given species of animal (Evans, 2018; Evans and Ganjam, 2017; Senger, 2012). The physiological processes involved in reproduction generally include the following: (1) gametogenesis (production of sperm or ova) and the pre- and peripubertal changes leading up to its onset; (2) release of gametes [i.e., sperm transport and maturation, penile erection and ejaculation of sperm (mammals), copulation between a male and a female of the same species (several vertebrate classes) and ovulation of oocytes]; (3) formation of the zygote (i.e., sperm storage, capacitation, and other processes leading to fertilization, or union, of a single sperm with an egg); (4) embryonic and fetal development during the incubation process in egg-bearing vertebrates or, especially in the case of mammals, during pregnancy (gestation) [i.e., activities related to the initiation and progression of zygote cleavage, blastocyst formation, separation of the germ layers, placentation (mammalian species), neurulation, and organogenesis (including sexual differentiation)]; (5) "birth" of a single or multiple offspring (hatching in oviparous vertebrates); and, finally, in mammalian species, (6) the initiation and maintenance of milk production (lactation) for the postpartum nutrition of offspring (Evans, 2018; Evans and Ganjam, 2017).

39.2.4 Reproductive toxicity

For the purposes of this chapter, "reproductive toxicity" will refer to any manifestations of xenobiotic exposure, including "endocrine disruption" (see discussion below), reflecting adverse effects on any of the physiological processes and associated behaviors and/or anatomical structures involved in animal reproduction or development (Fig. 39.1). This is a fairly broad definition which encompasses developmental toxicity, as well as any toxic effects of postpubertal exposures to xenobiotics on either male or female reproduction. "Developmental toxicity" refers to any adverse effect on the developing organism associated with either preconception parental exposures to toxicants or postconception xenobiotic exposures to the embryo, fetus, or prepubertal offspring, and adverse effects associated with developmental toxicity of xenobiotics might not necessarily be observed until after the affected individuals have reached sexual maturity (Eaton and Gilbert, 2013; Evans, 2018; Hodgson et al., 2000).

39.2.4.1 Teratogenesis

The term "teratogenesis" is derived from the Greek word for monster ("teras") and is a form of developmental toxicity (Rogers, 2013). "Teratogenesis" refers specifically to developmental defects induced by toxicant exposures occurring between conception and birth (Eaton and Gilbert, 2013; Evans, 2018; Hodgson et al., 2000; Rogers, 2013). The types of abnormalities that are typically associated with teratogenesis include embryonic or fetal death; morphological, functional, and/or neurobehavioral abnormalities; and decreased growth rate and/or birth weight (Evans, 2018; Rogers, 2013).

With respect to teratogenesis, there are six basic tenets of teratology, first defined by J.G. Wilson in 1959, which need to be kept in mind whenever gestational exposure to a teratogenic xenobiotic is suspected or when a chemical is being evaluated for its teratogenic potential (Evans, 2018; Wilson, 1977):

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
2. Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis.
4. The final manifestations of abnormal development are death, malformation, growth retardation, functional disorder.
5. The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).
6. Manifestations of deviant development increase in degree as dosage increases from no effect to the totally lethal level.

39.2.4.2 Mechanisms of reproductive toxicity and teratogenesis

In general, normal reproduction and development require rapidly replicating and differentiating cells undergoing mitosis and, within the gonads, meiosis. There are a wide range of specific mechanisms of action by which xenobiotics can adversely affect reproductive function, including embryonic and fetal development. Many of these mechanisms are the same as those for toxicants affecting other organ systems and essentially involve some sort of toxicant-induced interference with the cell cycle, cellular dysregulation, and alterations in cellular maintenance which, when possible, the body attempts to repair, either

successfully or unsuccessfully (Gregus, 2013). Oxidative damage and interference with normal enzymatic reactions are two common mechanisms by which xenobiotics and, especially, some CWAs (Smith et al., 2008) can cause the dysregulation and altered maintenance of cells within various organs and tissues.

Teratogenesis can be associated with each of the following mechanisms of action: (1) excessive cell death; (2) interference with apoptosis; (3) reduced cellular proliferation rate; (4) failed interactions between cells; (5) impaired morphogenetic movements; (6) reduced synthesis of components essential for growth and development; (7) mechanical disruption; and (8) alterations in pH (Evans, 2018; Hood, 2006; Hood et al., 2002). Some teratogens are capable of more than one mechanism of action, and it is important to keep in mind that the observed developmental abnormalities associated with exposure to any given teratogen will in large part be dependent on the timing of the exposure to that xenobiotic during gestation. Familiarity with the timing of important developmental events in species of interest is critical in the diagnosis and prevention of teratogenesis, as well as the design of experiments investigating the teratogenic potential of different chemicals in animal models (Evans, 2018).

Normal reproduction and development require signaling within and between a variety of diverse organs, and, in sexual reproduction and mammalian pregnancy, critical communication even takes place between distinctly different organisms (i.e., male and female and mother and offspring, respectively) (Evans, 2018). It should be remembered that premature parturition or abortion can be induced by any circumstances which cause fetal or, potentially, maternal stress and initiate the cascade of endocrine and neural signaling events which would normally lead to parturition (Evans, 2018). Any sublethal intoxication or emotionally traumatic event in a pregnant woman or animal has the potential to threaten fetal survival.

The dependence of reproductive function on signaling pathways inclusive of gene transcription makes this physiological process especially prone to adverse effects associated with xenobiotic-induced disruption of or interference with cell-to-cell, organ-to-organ, and/or even animal-to-animal communication. Many of the mechanisms which interfere in some way with physiological signaling activity can be classified as forms of "endocrine disruption" (see discussion below), but there is a great deal of overlap between the various different mechanisms for reproductive toxicity. The level of exposure to a particular toxicant is an important determinant of what toxic effects are observed, and xenobiotics which "disrupt" endocrine pathways can do so without interactions with endogenous receptors, using mechanisms of action which can cause other forms of toxic insult at various dosages.

39.2.4.3 Reproductive toxicants

Any xenobiotic associated with adverse effects on the development of male or female reproductive function can be classified as a "reproductive toxicant" (Evans, 2018; Rogers, 2013). Even chemicals adversely affecting animal well-being have the potential to have a negative impact on development and reproductive function. This chapter will attempt to focus on toxicants which are available for or could arise from military and terrorist activities and specific mechanisms of actions which have a direct effect upon the male and/or female reproductive tract or which target normal embryonic and/or fetal growth and maturation (Evans, 2018).

39.2.4.4 Teratogens

The subclass of reproductive substances capable of inducing teratogenesis is referred to as "teratogens." Some teratogenic chemicals induce their adverse effects "indirectly" on the fetus by altering maternal synthesis of essential nutrients or by other mechanisms which do not require their transport across the placenta. However, many teratogens "directly" affect fetal development by crossing the "placental barrier" and entering the fetal circulation. The passage of nutrients, hormones, and other endogenous, as well as exogenous, substances across the placenta has been traditionally thought of by some references as primarily a function of the intimacy (i.e., number of tissue layers) between the maternal and fetal circulations, especially with respect to maternal immunoglobulins which cross some types of placentation but not others (Evans, 2018). While it is true that placental characteristics, such as thickness, surface area, carrier systems, and lipid-protein characteristics, can influence the passage of xenobiotics across the placenta and that the placenta is generally impermeable to chemicals with molecular weights greater than 1000 Da, most xenobiotics have molecular weights less than 500 Da and cross the placenta by simple diffusion (Foster and Gray, 2013). It is currently thought that a potential teratogen's molecular size, degree of ionization, protein binding, and lipid solubility are the most important determinants of that chemical's ability to move from the maternal circulation across the placenta into the fetal circulation (Evans, 2018; Foster and Gray, 2013; Rozman and Klaassen, 2001). Some toxic xenobiotics can be actively transported by mechanisms intended for structurally similar endogenous molecules (Rozman and Klaassen, 2001), and there is some experimental evidence to suggest that transplacental transport of lead can mimic that of calcium (Evans, 2018; Evans et al., 2003).

39.2.4.5 Endocrine disruption

"Endocrine disruption" is a developing, multidisciplinary area of research, involving aspects of both toxicology and

endocrinology (McLachlan, 2001) and is a potential mechanism of action for many toxicants, especially those affecting reproduction. This term has been defined in a variety of different ways, depending on the circumstances and the intended audience. "Endocrine disruption" can also be defined fairly narrowly with respect to toxicant origin (synthetic versus naturally occurring); source or site of toxicant exposure (exposure from warfare- or terrorism-related activities versus exposure from environmental contamination); xenobiotic mechanism of action (receptor agonism and/or antagonism versus other mechanisms independent of direct interactions between xenobiotics and receptors); and/or the timing of exposure (prenatal versus postnatal exposures) (Evans, 2018; Krimsky, 2000, 2001). However, the definition of "endocrine disruption" used in this and other book chapters previously written by the author (Evans, 2017, 2018) will be fairly "broad" and will encompass the effects of any synthetic or naturally occurring xenobiotic which can affect the endocrine system of exposed individuals (i.e., the balance of normal hormonal functions) and, as a result of exposure, cause physiological alterations (Evans, 2017, 2018; Hodgson et al., 2000; Keith, 1997). Within the broad scope of this definition, reproduction, including prenatal and prepubertal development, certainly would be expected to be one of the physiological functions most profoundly affected by chemicals associated with chemical warfare or environmental contamination capable of endocrine disruption; however, adverse effects on other, "nonreproductive" endocrine systems can also be associated with exposures to xenobiotics (Evans, 2018; Guillette, 2006). Thyroid function, glucocorticoid metabolism, and other endocrine as well as enzymatic factors associated with adipogenesis have recently been shown to be susceptible to interference by several different classes of chemical compounds (Capen, 2008; Cooke and Naz, 2005; Evans, 2017, 2018; Guillette, 2006; Grün and Blumberg, 2006; Newbold et al., 2005, 2006).

39.2.4.6 Mechanisms of endocrine disruption

Although the imitation and/or inhibition of the actions of androgens and, especially, estrogens by xenobiotics is what was first referred to as "endocrine disruption," both the multidisciplinary area of study and mechanism of action generally referred to as "endocrine disruption" have evolved over the years to encompass a wide range of specific mechanisms of action which can ultimately result in adverse effects on invertebrate and/or vertebrate animals (Evans, 2017, 2018; McLachlan, 2001). Endocrine disruption involves many mechanisms of action which can ultimately result in adverse effects on animal species. The mechanisms of action involved in endocrine disruption can include effects which are mediated directly by interactions between the xenobiotic and an endogenous hormone receptor (i.e., the xenobiotic functions as a ligand for an endogenous receptor and a receptor−ligand complex is formed), as well as those adverse effects which alter hormonal functions without direct interactions between the toxicant and an endogenous receptor (Capen, 2008; Evans, 2017, 2018; Keith, 1997). It should also be noted that a given xenobiotic can potentially disrupt the normal balance of hormonal function by more than one mechanism which is independent of direct interactions between the toxicant and an endogenous hormone receptor (Evans, 2017, 2018).

"Classic" endocrine disruption can involve imitation or mimicry of the interactions between cellular receptors and endogenous hormones (i.e., receptor agonism) and/or a blockade or inhibition of the formation of receptor-hormone complexes (i.e., receptor antagonism) (Evans, 2017, 2018; McLachlan, 2001), and both genomic and nongenomic physiological responses can be affected by this mimicry or blockade of endogenous hormone receptor-mediated activity (Evans, 2017, 2018; Thomas and Khan, 2005). Endocrine disruption can also be mediated by the complex interactions between the endogenous aryl hydrocarbon receptor (AhR) and its major agonists, which are xenobiotics belonging to the class of environmental contaminants referred to collectively as "halogenated" or "polyhalogenated aromatic hydrocarbons" (HAHs or PAHs, respectively) (Evans, 2017, 2018; Safe, 2005).

Endocrine disruption. which is independent of interactions between xenobiotics and endogenous hormone receptors, can occur in a variety of different ways, including alterations in the number of hormone receptor sites (up- or downregulation) or direct or indirect hormone modifications which alter hormonal function (Evans, 2017, 2018; Keith, 1997). Xenobiotics can change the rate of synthesis or destruction of endogenous hormones and can alter how hormones are stored, how they are released into and/or transported within the circulation, or even how they are eventually cleared from the body (Capen, 2008; Evans, 2017, 2018; Keith, 1997; Sikka et al., 2005). Any xenobiotic toxic to hormone-producing organs or tissues (e.g., testis and ovary) also has the potential to decrease hormone synthesis and thereby indirectly cause endocrine disruption (Devine and Hoyer, 2005; Evans, 2017, 2018).

In addition to the aforementioned mechanisms of endocrine disruption, there has recently been increasing interest in the association between prenatal exposures to some hormonally active toxicants and the postnatal development of neoplasia (cancer) involving the reproductive tract, as well as the occurrence of transgenerational or vertically transmitted adverse reproductive effects (Crews and McLachlan, 2006). Either "genetic" mutations [i.e.,

alterations in the genotype or deoxyribonucleic acid (DNA) sequence] or "epigenetic" changes, such as DNA methylation of CpG nucleotides in the promoter regions of genes, which are heritable but nongenetic modifications in the properties of a cell (inherited phenotypic alteration without genotypic change) are possible explanations for these phenomena (Crews and McLachlan, 2006; Evans, 2017, 2018; McLachlan, 2001). Patterns of DNA methylation are generally established during development at the gastrulation stage (i.e., lineage-specific pattern in somatic cells) and after sex determination (i.e., germ line-specific lineage pattern in the gonad), and DNA methylation can facilitate "genomic imprinting," which results in the expression of the allele from only one parent (i.e., monoallelic expression) (Anway and Skinner, 2006; McLachlan, 2001).

39.2.4.7 Endocrine-disrupting chemicals, endocrine disruptors, and hormonally active agents

Any reproductive toxicant capable of endocrine disruption can also be considered an "endocrine-disrupting chemical" ("EDC") or an "endocrine disruptor." Another term frequently used with respect to endocrine disruption, especially regarding xenobiotics which interact with endogenous hormone receptors, is "hormonally active agent" or "HAA." In most instances, "endocrine-disrupting chemical," "endocrine disruptor," or "hormonally active agent" can be used interchangeably to discuss the actions of a given xenobiotic (Evans, 2017, 2018).

39.3 The reproductive toxicity of selected toxicants

It should be remembered that the use of CWAs will invariably be associated with wars, acts of terrorism, revolutions, and civil unrest, all of which do not occur in a vacuum and will be concurrent with emotional stress, famine, and other conditions leading to reproductive failure. As a result, information is relatively lacking with respect to specific, adverse reproductive effects, as well as endocrine disruption, related to acute and, particularly, low-level, chronic exposures to many potential CWAs. In addition, much of the information which does exist regarding the long-term reproductive and teratogenic effects of these types of weapons is, unfortunately, somewhat contradictory. While it is possible that exposures to some CWAs might not generally be associated with serious, adverse reproductive effects, the discrepancies between the results of different studies are probably, in part, due to variability in individual responses to specific toxicants and the complexity of analyses of human epidemiological data. In addition, differences in dosing regimens, routes of exposure, and the animal models utilized for in vivo studies can confound comparisons of experimental results. The relative insensitivity of many reproductive endpoints might also contribute to the impression that some chemical weapons do not adversely affect reproductive function.

The embryo and fetus, without a developed blood−brain barrier and with only rudimentary DNA repair mechanisms and hepatic detoxifying and metabolizing capabilities, are especially susceptible, as compared to adults, to the adverse effects of low-level exposures to xenobiotics (Evans, 2017, 2018; Newbold et al., 2006). There has recently been increasing concern within the regulatory, public health, and scientific communities about the effects of prenatal exposures to potential reproductive toxicants, especially those capable of endocrine disruption, on humans and animals. In order to increase our understanding of the long-term, adverse reproductive effects associated with warfare and acts of terrorism, public knowledge of the use of CWAs or awareness of massive releases of reproductive toxicants associated with confirmed acts of terrorism should result in increased surveillance for phenotypic abnormalities in the most susceptible populations of humans and animals exposed during embryonic, fetal, and early postnatal development. Based on these epidemiological observations, carefully designed experiments mimicking "real-life" exposures can be performed in multiple laboratory animal species, using sensitive biomarkers of toxic insult to reproductive development and function, to further elucidate the adverse reproductive effects of xenobiotic exposures likely to be associated with military and/or terrorist activities.

Our relative lack of understanding of the effects of CWAs and other xenobiotics on reproductive function can also be attributed to the complexity of the entire reproductive process and the mechanisms by which it is regulated. It should be evident from Fig. 39.1 that maximum reproductive efficiency, including normal embryonic and fetal development, is dependent on the structural and functional integrity of multiple organs and tissues, as well as various physiological processes and signaling pathways within and (with respect to sexual reproduction and pregnancy) between individuals. Putative reproductive toxicants can affect one or several different steps in the reproductive process, depending on the physiological timing, duration, and level of exposure (Evans, 2018; Foster and Gray, 2013).

In "real life," humans and animals can be exposed to some toxicants both pre- and postnatally. Many organic xenobiotics have the potential to bioaccumulate within exposed individuals, possibly affecting future generations by way of genetic and epigenetic effects. However, reproductive endpoints, such as conception rates and sperm counts, are relatively insensitive, and subtle, toxicant-

induced changes in reproductive efficiency can be overlooked or missed (Evans, 2018).

Much of the evidence for the adverse reproductive effects of selected toxicants will be based on cases involving wildlife exposures to environmental contaminants or on the experimental results of research exposing laboratory animals to large, "pharmacological" doses of potential toxicants. When available, data will be presented from accidental or intentional human and domestic animal exposures to toxicants associated with riot control and chemical warfare or with "environmental catastrophes" where incidences of infertility, abortion, and teratogenesis have been traced over the course of a number of years.

From an epidemiological perspective, it can be extremely challenging to determine the exact cause of reproductive abnormalities in humans and animals. Questions will often remain as to whether the observed poor reproductive outcomes associated with acute exposures to toxicants are due to direct effects of these chemicals on reproductive function or are secondary to toxicant-induced systemic disease and its accompanying stress (e.g., abortions and preterm births in intoxicated, pregnant women or animals). There are multiple factors, including exposures to mixtures of toxicants and other concurrently occurring causes of reproductive failure, which need to be taken into consideration in heterogeneous populations where exposures to toxic agents are not uniform between individuals. This is especially true in instances when there is a significant time interval between exposure to potential toxicants and the observed reproductive outcomes.

39.3.1 The reproductive toxicity of riot control agents

The major lacrimatory and irritant riot control agents include α-chlorbenzylidene malonitrile (CS), dibenz (b,f)-1:4 oxazepine (CR), ω-chloroacetophenone (CN), and oleoresin of capsicum (OC pepper spray) (Salem et al., 2008a). Exposure of pregnant women or animals to these compounds could be expected to be associated with maternal and/or fetal stress, which could potentially lead to the induction of premature parturition (Evans, 2018). Although the riot control agents CS and CN are both alkylating agents, with at least the potential to adversely affect embryonic and fetal development, neither of these chemicals has yet been found to be embryotoxic or teratogenic (Salem et al., 2008a; Sanford, 1976). Limited studies performed with laboratory animals suggest that CR is neither embryotoxic nor teratogenic and that OC, with the possible exception of a slightly reduced crown−rump length, is not associated with any adverse effects on reproductive function (Salem et al., 2008a).

39.3.2 The reproductive toxicity of chemical warfare agents

While it would seem logical that participation in wars would not be conducive to optimal reproductive function, there are conflicting data regarding the short-term and long-term effects of suspected CWA exposure on male and female fertility (Abu-Musa et al., 2008). Additionally, given current global politics and the "War on Terrorism," pertinent information regarding many "newer" potential CWAs is very likely to be unavailable for public review. In order to be as complete as possible, some of the more "historical" and currently available CWAs will be discussed in the context of their primary, immediate, adverse effects on humans and animals. By organizing this discussion in that manner, the potential adverse reproductive effects of "new" CWAs can be anticipated based on the similarities of those toxicants to existing chemical weapons. For simplicity, CWAs will be broadly classified as vesicants (i.e., blistering agents), inhibitors of protein synthesis (e.g., ricin), inhibitors of cellular respiration or "blood agents" (e.g., hydrogen cyanide and cyanide-related compounds), and nerve agents (i.e., organophosphate compounds).

39.3.2.1 Vesicants

39.3.2.1.1 Arsenicals

While arsenicals will be discussed in this chapter with respect to their use in chemical warfare, the metalloid element arsenic, which is classified as a carcinogen, is also a potential environmental contaminant. Arsenicals continue to have important industrial and agricultural uses, and arsenic-containing feed additives, pesticides, and wood preservatives are all still readily available. In general, arsenic binds to sulfhydryl groups, with the activities of thiol-containing enzymes, including those involved in cellular energy production, frequently being adversely affected (Wismer, 2018). Increased capillary permeability is also associated with many acute intoxications involving arsenic.

It is important when discussing the toxicity of arsenicals to distinguish between the effects of organic and inorganic forms of arsenic. Several organic arsenicals, including lewisite [dichloro (2-chlorovinyl) arsine or Agent L], diphenylcyanoarsine, and diphenylchloroarsine, have been used as vesicants and systemic toxicants (Ishii et al., 2004; Wismer, 2018). Like other arsenicals, lewisite has been associated with fetal death in laboratory animals; however, lewisite is reported to not be teratogenic (Wismer, 2018). In contrast, a degradation product of diphenylcyanoarsine and diphenylchloroarsine, diphenylarsinic acid, has recently been associated with abnormal brain development in humans (Ishii et al., 2004).

Inorganic forms of arsenic, particularly arsenite and arsenate, have been associated with neoplasia, estrogenic activity, and testicular and ovarian toxicity, as well congenital neural tube, skeletal, and gonadal abnormalities, in laboratory rodents (Golub, 2006b). Epidemiological evidence in human populations has suggested that acute arsenic exposures with sublethal, maternal toxicity and subchronic exposures to elevated arsenic concentrations in drinking water have both been associated with increased incidences of abortions, stillbirths, and preterm deliveries (Golub, 2006b).

39.3.2.1.2 Chlorine gas

Chlorine (Cl_2) is one of the more commonly produced chemicals in the United States, and chlorine gas is a potent oxidant which is very irritating and, potentially, corrosive (Kikilo et al., 2008; Smith et al., 2008; Wismer, 2018). Chlorine gas is used as a pulmonary and choking agent, and exposure is frequently associated with moderate to severe, painful irritation of the eyes and respiratory tract (Wismer, 2018). Such stressful, sublethal exposures in late-gestational women or animals might be expected to be associated with the induction of premature parturition and, possibly, abortion. Oxidative stress can definitely have adverse effects on reproductive function, but the chronic disease associated with chlorine gas exposure is primarily related to the ocular and respiratory systems (Smith et al., 2008). The limited information available on the reproductive effects of chlorine gas indicates that it is teratogenic (Wismer, 2018).

39.3.2.1.3 Phosgene and phosgene oxime

The most important industrial use of phosgene (Agent CG or carbonyl chloride) is in the production of isocyanates (Kikilo et al., 2008), and MIC exposure is discussed with respect to its accidental release in Bhopal, India. Phosgene is classified as a choking agent, and it acylates sulfhydryl, amine, and hydroxyl groups. Phosgene oxime (Agent CX), a halogenated oxime, is a nonpersistent, chemical blistering agent, which, like phosgene, interacts with sulfhydryl and amine groups (Wismer, 2018). Other than possible adverse reproductive outcomes related to maternal and/or fetal stress or increased dermal absorption of concurrently used CWAs (Wismer, 2018), phosgene oxime is unlikely to have direct adverse effects on reproductive function.

39.3.2.1.4 Sulfur mustard

With respect to sulfur mustard, as well as other vesicants, and even in instances of occupational exposures to pesticides, the genital area, especially the scrotum, is well-recognized as being thin-skinned, with a high degree of cutaneous diffusivity, both characteristics which facilitate enhanced cutaneous absorption of xenobiotics and rapid onset of clinical signs (Lehman-McKeeman, 2013; Panahi et al., 2013). Sulfur mustard [*bis*-(2-chloroethyl) sulfide; mustard gas, Agents HD, H or HS] and analogs, such as 2-chloroethyl ethyl sulfide (CEES), are vesicants which can damage cells by alkylation of macromolecules (i.e., DNA, ribonucleic acid and proteins), oxidative stress, glutathione depletion, and inflammation (Dacre and Goldman, 1996; Smith et al., 2008; Watson and Griffin, 1992; Wismer, 2018). Similar to ionizing radiation and a variety of other radiomimetic alkylating agents, such as busulfan, cyclophosphamide, and nitrogen mustard, sulfur mustard and its analogs can target rapidly dividing cells in multiple organs, including the testes and ovaries, as well as in the developing embryo and fetus (Foster and Gray, 2013; Hurst and Smith, 2008; Rogers, 2013; Wismer, 2018). Consistent with DNA alkylation, as well as, possibly, other mechanisms of action, men exposed to sulfur mustard gas have been reported to have lower sperm counts and testosterone concentrations for several weeks following respiratory exposure. Acute and chronic effects on male fertility have also been reported with cutaneous exposure to sulfur mustard in the genital region, but these results are not consistent between studies (Panahi et al., 2013). Additionally, Iraqi use of mustard gas has been associated with alterations in the infant sex ratio and an increase in some birth defects in children (Arnetz et al., 2013; Azizi et al., 1995; Pour-Jafari, 1994; Wismer, 2018).

However, despite epidemiological and laboratory animal evidence indicating that sulfur mustard is a teratogen and reproductive toxicant in humans and animals (Azizi et al., 1995; Panahi et al., 2013; Pour-Jafari, 1994; Wismer, 2018), contradictory research data involving several different routes of exposure have suggested no adverse, sulfur-mustard-related reproductive effects, especially at levels of exposure not associated with maternal intoxication (Dacre and Goldman, 1996; Watson and Griffin, 1992; Wismer, 2018). While, based on its mechanisms of action, it appears that exposures to sulfur mustard should be associated with several, potential adverse reproductive outcomes, including maternal and/or fetal stress-induced premature parturition, additional mustard gas inhalation experiments should be performed in rodent and nonrodent animal models to confirm this suspicion.

39.3.2.2 Inhibitors of protein synthesis

39.3.2.2.1 Ricin

The seeds of the ubiquitous castor bean plant (*Ricinus communis*) contain high concentrations of a highly toxic, relatively stable, heterodimeric, glycoprotein toxin, ricin, which is a type 2 ribosome-inactivating protein (RIP) (Burrows

and Tyrl, 2001; Millard and LeClaire, 2008; Salem et al., 2008b). The inhibition of protein synthesis by ricin and related type 2 RIPs has been associated with endothelial toxicity, and, depending on the route of exposure, severe gastrointestinal or respiratory disease and death (Millard et al., 2008). While it would be anticipated that acute, sublethal ricin intoxication would be associated with abortion or preterm delivery secondary to maternal and/or fetal stress, ricin has also been shown to have direct adverse effects on reproductive function in female rabbits, causing abortion and inhibition ovulation and implantation in this species (Salhab et al., 1999). Because of the availability of the raw ingredients and the relative ease of its extraction, ricin has the potential to be a "low-tech" alternative for terrorist attacks targeting public water supplies (Salem et al., 2008b). Additional research is needed in order to have a better idea of the adverse reproductive effects which would be anticipated with sublethal and/or chronic exposures to ricin and related toxins (Millard et al., 2008).

39.3.2.3 Inhibitors of cellular respiration ("blood agents")

39.3.2.3.1 Hydrogen cyanide and cyanide-related compounds

With respect to their use in chemical warfare and, most likely, acts of terrorism, hydrogen cyanide [Agent AC, hydrocyanic acid (liquid form) or prussic acid], cyanogen halides and other cyanide-related compounds are frequently described as a "blood agents" (Kikilo et al., 2008; Wismer, 2018). The major mechanism of action associated with acute cyanide intoxication is the formation of a stable complex with the ferric iron (Fe^{3+}) in cytochrome oxidases, resulting in cytotoxic hypoxia from the inhibition of cellular respiration, oxygen utilization, and energy production (Ballantyne and Salem, 2008; Wismer, 2018). As a result of the rapid lethality of this class of compounds, very few studies have been conducted to investigate the adverse reproductive effects of hydrogen cyanide and cyanide-related compounds (e.g., cyanogen halides, cyanides, and nitriles). However, there is evidence to suggest that cyanide exposure in laboratory animals and livestock exposures to plants containing cyanogenic glycosides can be associated with embryonic and fetal death, as well as teratogenesis (Ballantyne et al., 2008; Burrows and Tyrl, 2001; Wismer, 2018). In addition, it would be anticipated that sublethal cyanide-induced hypoxia could cause enough maternal and/or fetal stress to result in abortion or preterm deliveries.

39.3.2.4 Nerve agents

39.3.2.4.1 Organophosphate nerve agents

The development of easily disseminated nerve agents as chemical weapons has been of interest to both military strategists and terrorist organizations because of the ability of these chemicals to rapidly incapacitate and kill opposing forces, as well as civilian populations. The major chemical nerve agents, tabun (Agent GA), sarin (Agent GB), soman (Agent GD), cyclosarin (Agent GF), and Agent VX are extremely toxic, and even very brief exposures to these nerve agents can be lethal (Kikilo et al., 2008; Watson et al., 2006; Wismer, 2018). These chemicals are classified as organophosphorus, organophosphate, or "OP" compounds, and their mechanism of action involves the competitive and irreversible inhibition of acetylcholinesterase (AChE) (Gupta et al., 2018; Watson et al., 2006; Wismer, 2018). Commonly used OP and carbamate pesticides also inhibit AChE, and these chemicals, although generally less potent than those designed for use as CWAs, usually have longer lasting effects and have the potential to be used as "low-tech" chemical weapons.

There have been some discrepancies between the results of various studies evaluating the reproductive toxicity of different OP compounds. While decreased libido has been observed in men following acute exposures to both OP nerve agents and insecticides, this "reproductive" effect is most likely related to the neurobehavioral effects, such as posttraumatic stress disorder, associated with acute exposures to these chemicals (McDonough and Romano, 2008). Some nerve agents have been associated with postimplantation morbidity and mortality in laboratory animals (Wismer, 2018), but rats and rabbits exposed to soman did not apparently experience fetal toxicity or prenatal mortality, even with maternal illness (Wismer, 2018). Similarly, low-level exposures to other nerve agents, as well as some insecticides, have not consistently resulted in impaired fertility and developmental abnormalities (McDonough et al., 2008). However, adverse reproductive effects have recently been reported by several authors in association with OP insecticide exposures (Joshi et al., 2007; Peiris-John and Wickremasinghe, 2008; Sikka and Gurbuz, 2006). Chlorpyrifos exposure has resulted in decreased sperm counts and testosterone concentrations, as well as testicular degeneration, in laboratory animals (Joshi et al., 2007). Similar abnormalities have also been observed in humans following low-level exposures to OP insecticides (Peiris-John and Wickremasinghe, 2008; Sikka and Gurbuz, 2006). It has been reported that OP insecticides can cause disturbances in the feedback loops within the hypothalamic−pituitary−adrenal axis, thereby affecting the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Sikka and Gurbuz, 2006), and some members of this class of pesticides have even been reported to have antiandrogenic activity (Kitamura et al., 2006). In addition, exposures to OP insecticides can also cause oxidative stress, leading to increased apoptosis within the testes (Sikka and Gurbuz, 2006).

As mentioned previously, maternal and/or fetal stress associated with sublethal exposures to OP insecticides could lead to an increased incidence of abortions or preterm births in intoxicated, pregnant women and animals. Because of lower levels of detoxifying enzymes (i.e., paraoxonase), the fetus appears to be more susceptible to OP intoxication than adults, and developmental neurotoxicity and growth retardation have been associated with low-level, prenatal exposures of humans to OP insecticides (Desaiah, 1998; Eskenazi et al., 2008; Peiris-John and Wickremasinghe, 2008). In addition to the maternal and fetal effects, OP insecticides can also have direct toxic effects on the placenta, possibly involving (depending on the species) AChE inhibition within the placental cholinergic system (Pelkonen et al., 2006).

Since the effects of OP insecticides are generally longer lasting and more diverse than those of OP nerve agents, extrapolations between OP insecticides and nerve agents need to take into consideration the toxicokinetic and toxicodynamic differences between these two classes of OP compounds (McDonough et al., 2008). Given their frequent occupational use and the duration of the toxic effects of OP insecticides, it is probably more likely that adverse reproductive outcomes will be investigated and observed with exposures to these compounds than with exposures to nerve agents specifically designed for immediate incapacitation without environmental persistence. Depending on the circumstances, acute or low-level exposures to OP nerve agents might not be associated with overt effects on reproductive function, especially when direct, toxicant-induced effects on reproductive performance are of secondary importance to neurological and/or psychological concerns and the reproductive endpoints being assessed are relatively insensitive. Duration, route, and amount (i.e., dose) of nerve agent exposure, the developmental period during which exposure occurs, and the reproductive endpoints and animal species being evaluated are factors which will need to be taken into consideration in the design of future studies investigating the direct effects of nerve agents on fertility and embryonic and fetal development.

39.3.2.5 The reproductive toxicity environmental contaminants resulting from acts of terrorism

Not all acts of "chemical warfare" are going to necessarily involve weapons specifically designed for that purpose. Especially in instances of terrorism or military attacks involving nongovernmental militias or governments with limited weapons resources, chemical warfare can be "low tech" and make use of toxic chemicals in commercially available products or those hazardous materials present at manufacturing, processing, or storage facilities. Ionizing radiation can be released into the environment through strategic military use of nuclear weapons or from "nuclear accidents" involving municipal power plants, nuclear-powered aircraft carriers and submarines or the explosion of misplaced, misused, or stolen nuclear "weapons of mass destruction." In addition, many potential reproductive toxicants, including pesticides, are routinely used in various manufacturing and agricultural processes. Acts of terrorism or military strikes on industrial or agricultural complexes have the potential to greatly increase the exposure of humans and animals to a large number of different toxicants in the air, water, soil, and food chain.

Depending on the circumstances, both short- and long-term (i.e., immediate and delayed) morbidity and mortality can be observed in "environmental disasters." In these types of catastrophic events, illness and death can be due to the direct effects of toxicants, or they can arise secondarily from other factors associated with the environmental release of xenobiotics. Related conditions, such as famine, can accompany disasters and negatively impact reproductive function in humans and animals. The very real or, in some cases, imagined or exaggerated threats to human and animal welfare from environmental contaminations can be associated with instances of mass fear, panic, and "emotional incapacitation." These psychological stresses can affect reproductive function and, especially, gestational length and fetal survival in humans and animals. Emotional responses are naturally heightened with toxic exposures involving pregnant women and children.

39.3.2.6 Ionizing radiation

Ionizing radiation can target rapidly dividing cells in multiple organs, including the testes and ovaries, as well as the developing embryo and fetus (Cockerham et al., 2008; Foster and Gray, 2013; Rogers, 2013). Exposure of males to ionizing radiation can result in diminished spermatogenesis and testosterone production by the testes, with increased secretion of LH and FSH by the anterior pituitary (Cockerham et al., 2008). Consistent with these effects, Ukrainian workers involved in the cleanup of radioactive materials after the Chernobyl nuclear accident had increased ultramorphological sperm abnormalities (Cockerham et al., 2008; Fischbein et al., 1997). Similar to the radiation-induced endocrine effects observed in the testes, ovarian steroid production is reduced by exposure to ionizing radiation (Cockerham et al., 2008). Depending on the timing and dose of the radiation exposure, ionizing radiation can cause pubertal failure, ovarian failure, or premature menopause in women (Cockerham et al., 2008).

Clusters of Down syndrome in Belarus 9 months following the explosion at the Chernobyl nuclear power

plant suggest a radiosensitive phase of oogenesis in mammals around the time of ovulation and conception (Zatsepin et al., 2007). High radiation exposure in late-gestational women or pregnant animals has the potential to cause abortion or preterm births associated with maternal and/or fetal radiation sickness and stress. Depending on the stage of development and the dose of radiation, exposure of the conceptus, embryo, or fetus to ionizing radiation can result in lethality or morphologic abnormalities (Cockerham et al., 2008), and observations in humans and animals after the Chernobyl incident are consistent with these developmental effects (Østerås et al., 2007; Peterka et al., 2007). In addition, anxiety associated with exposures of pregnant women to ionizing radiation from the Chernobyl nuclear accident reportedly led to increased incidences of induced abortions in several European countries, even in instances where the exposure was minimal (Cordero, 1993).

39.3.2.7 Pesticides and other organic contaminants

Pesticides and other organic contaminants are ubiquitous in both industrial and agricultural settings. Acts of terrorism have the potential to increase the exposure of humans and animals to these types of xenobiotics in the environment by targeting industrial and agricultural complexes. The massive release of pesticides, in particular, has the potential to be a readily available means of inciting fear and inducing morbidity and mortality in humans and animals. In fact, as mentioned previously, carbamate and OP insecticides have the same basic mechanism of action as the previously discussed AChE-inhibiting nerve agents, and MIC, an intermediate in the production of carbamate insecticides, contains a cyanide moiety.

There have been many, well-documented instances of reproductive abnormalities in species of wildlife living in environments contaminated by a wide range of industrial and/or agricultural chemicals (Evans, 2018; Guillette, 2006; Hess and Iguchi, 2002; Jobling and Tyler, 2006; McLachlan, 2001; McLachlan et al., 2006). Wildlife populations are very likely sentinels for exposure to reproductive toxicants because of the contamination of the aquatic habitats in which many of them live and the bioaccumulation of some organic chemicals in predators (Hess and Iguchi, 2002). There is also recent evidence to suggest that domestic animals can act as potential sentinels for human exposure to endocrine disruptors and that hyperthyroidism in cats might be associated with exposure to polybrominated diphenyl ethers (PBDEs) (Dye et al., 2007).

Based on the observations of reproductive toxicity (including endocrine disruption) in wildlife and domestic animals, as well as ongoing concerns about reproductive dysgenesis in human populations and the observed effects of industrial accidents involving MIC and dioxins, there has been increasing interest in the effects of prenatal exposures of humans to suspected endocrine disruptors and other reproductive toxicants. However, when impaired reproductive function is discovered in adults, it is difficult to comment with complete certainty on the relative contributions of pre- versus postnatal exposures to reproductive toxicants. There is a wide array of pesticides and other organic environmental contaminants which have the potential to adversely impact reproductive function. Specific epidemiological or laboratory studies suggesting adverse reproductive effects of exposures to these xenobiotics will be discussed with respect to observed abnormalities in male and female reproductive function, as well as embryonic and fetal development.

39.3.2.7.1 Adverse effects of pesticides and other organic contaminants on male reproductive function

"Androgynization" or a state of indeterminate sexual development encompasses both feminization and demasculinization in males and, similar to the testicular dysgenesis syndrome described in humans, has been observed in populations of various vertebrate populations, including fish, amphibians, reptiles, birds, and mammals (Edwards et al., 2006; Evans, 2018). Adult and immature amphibians exposed to the herbicide atrazine and hatchling, juvenile, and adult male alligators originating from a lake previously contaminated with DDT and other persistent, bioaccumulated pesticides have been reported to exhibit varying patterns of androgynization (Evans, 2018; Hayes et al., 2006; Milnes et al., 2006). Although still somewhat controversial, there is evidence to support the observation that sperm counts in men within some industrialized regions of the world have been decreasing over the last several decades (Jørgensen et al., 2006; Skakkebæk et al., 2006; Swan et al., 2000). The findings of epidemiological studies have suggested a relationship between decreased anogenital distance and prenatal exposures of male infants and phthalates used as plasticizers, as well as a correlation between reduced semen quality in men within certain regions of the United States and the metabolites of several economically important herbicides (Swan et al., 2003a,b, 2005). In addition, a recently completed epidemiological study in Italy has demonstrated a significant relationship between postnatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and abnormal semen and endocrine parameters in men (Mocarelli et al., 2008).

The dicarboximide fungicides, vinclozolin and procymidone, and/or their metabolites inhibit the binding of androgens to nuclear androgen receptors and can demasculinize and feminize the prenatally exposed male fetus or induce important alterations in pre- or peripubertally

exposed offspring (Evans, 2017, 2018; Gray et al., 2006; Monosson et al., 1999). While still subject to debate amongst scientists, vinclozolin has also been reported to be capable of inducing epigenetic modifications which facilitate the occurrence of transgenerational or vertically transmitted reproductive abnormalities (Anway et al., 2005; Anway and Skinner, 2006). Linuron, *p,p′*-DDE, prochloraz, PBDEs, and selected OPs can function as androgen receptor antagonists (Gray et al., 2006; Kitamura et al., 2006), and AhR-mediated effects of TCDD can interfere with the biosynthesis of testosterone and disrupt testosterone signal transduction pathways (Jana et al., 1999; Mocarelli et al., 2008; Sikka et al., 2005).

The testes have xenobiotic biotransformation capabilities within both Leydig and Sertoli cells (Thomas and Thomas, 2001). While many toxicants and/or their metabolites are capable of producing relatively nonspecific effects, such as oxidative stress, there are a number of pesticides and other organic compounds which target specific cell populations within the testes. Several toxicants targeting Sertoli cells, including diethylhexyl phthalate and 2,5-hexanedione (a metabolite of *n*-hexane), have age- and species-specific effects (Creasy and Foster, 2002; Foster and Gray, 2013; Thomas and Thomas, 2001). Tri-*o*-cresyl phosphate, an industrial chemical used in lacquers and varnishes and associated with some organophosphate insecticides, inhibits LH-induced steroidogenesis in Leydig cells but, after Leydig cell-mediated conversion to its active metabolite, causes morphological abnormalities in Sertoli cells (Creasy and Foster, 2002; Evans, 2018; Thomas and Thomas, 2001).

As mentioned previously with regards to sulfur mustard, a variety of radiomimetic alkylating agents, including busulfan, cyclophosphamide, and nitrogen mustard, can target rapidly dividing mitotic or meiotic germ cells in the testes. In some instances, xenobiotics can target a specific population of germ cell precursors, such as spermatogonia spermatocytes or round or elongate spermatids (Creasy and Foster, 2002; Evans, 2018; Foster and Gray, 2013). TCDD appears to adversely affect several populations of spermatozoal precursors and alters the sex ratio in favor of female offspring (i.e., decreased viability of y chromosome-bearing sperm) (Foster and Gray, 2013; Ishihara et al., 2007; Mocarelli et al., 2008; Thomas and Thomas, 2001).

39.3.2.7.2 Adverse effects of pesticides and other organic contaminants on female reproductive function

A wide range of agricultural and industrial chemicals have estrogenic and/or antiestrogenic activities (Evans, 2018), and some of the synthetic xenobiotics most commonly discussed with respect to these activities include DDT, polychlorinated biphenyls, and TCDD (McLachlan, 2001). Effluents from industrial and agricultural activities have been shown to have androgenic activities and are associated with the masculinization of female fish (Evans, 2018; Gray et al., 2006; Orlando et al., 2004).

The effects of toxicants on specific cell types within the ovaries are not as well understood as they are in the testes (Thomas and Thomas, 2001). Many female reproductive toxicants do not actually target particular cell lines but, rather, disrupt the endocrine milieu of the tubular genitalia or cause changes in ovarian structures secondary to alterations in the hypothalamic−pituitary−gonadal axis (Yuan and Foley, 2002). Like the testes, the ovaries also have some xenobiotic biotransformation capabilities, and oxidative damage can adversely affect ovarian structure and function (Thomas and Thomas, 2001; Yuan and Foley, 2002). Phthalates and TCDD can delay or decrease ovulations, and, like sulfur mustard and ionizing radiation, some of the alkylating agents reported to adversely affect rapidly dividing germ cells within the testes can also adversely affect primordial follicles within the ovary (Devine and Hoyer, 2005; Thomas and Thomas, 2001). Several PAHs [i.e., BaP, 3-methylcholanthrene (3-MC) and DMBA] and 1,3-butadiene appear to target oocytes in preantral follicles, and DMBA can adversely affect antral follicular development (Devine and Hoyer, 2005; Evans, 2018).

39.3.2.7.3 Adverse effects of pesticides and other organic contaminants on embryonic/fetal development

As has been emphasized previously, the developing fetus undergoing phenotypic sexual differentiation is particularly susceptible to the adverse effects of various agonists and antagonists of estrogen and androgen receptors (Evans, 2017, 2018; Hess and Iguchi, 2002). A large number of xenobiotics, including many pesticides (e.g., carbamate, OP, organochlorine and pyrethroid insecticides) and other potential organic environmental contaminants, have been recognized as potential teratogens in humans and animals (Desaiah and Chang, 1998; Evans, 2017, 2018; Rogers, 2013). Alkylating agents with radiomimetic activity, such as busulfan, cyclophosphamide, and nitrogen mustard, cause teratogenesis by targeting rapidly replicating cells (Rogers, 2013). TCDD has been found to be teratogenic in both laboratory animals exposed experimentally (Aragon et al., 2008; Kransler et al., 2007) and in humans exposed following an accidental release in Seveso, Italy, in 1976 (Alaluusua et al., 2004). Accidental exposure to MIC in Bhopal, India, in 1984 resulted in a significant increase in spontaneous abortions and neonatal

mortality in humans (Varma, 1987; Varma and Mulay, 2006), and these epidemiological data were corroborated by the results of a subsequent rodent experiment (Varma, 1987). In addition, some xenobiotics can cause fetal death and abortion by having direct toxic effects on the placenta, rather than on the fetus itself (Gupta, 2007; Pelkonen et al., 2006).

39.3.2.8 Heavy metals

Heavy metals are routinely used in various manufacturing processes and are contained within many products commonly used by humans. Acts of terrorism have the potential to increase the environmental exposure of humans and animals to heavy metals by targeting industrial complexes and sewage treatment facilities. Because a number of heavy metals have the potential to affect different stages of reproductive function by different mechanisms of action, the adverse effects of metals on male and female reproduction and embryonic and fetal development will be discussed separately.

39.3.2.8.1 Adverse effects of heavy metals on male reproductive function

Excessive cobalt can potentially interfere with normal spermatogenesis, and even generalized hypoxia related to increased blood viscosity which affects the testes (Evans, 2018; Thomas, 1995). Chromium and vanadium have also been associated with adverse reproductive effects, and cis-platinum exposure has been associated with the death of spermatocytes and spermatids, as well as disruption of Sertoli cell tight junctions (Evans, 2018; Thomas, 1995; Thomas and Thomas, 2001). Exposure of male laboratory animals to organotin compounds has been associated with reduced testicular size, alterations in testicular morphology, and impaired spermatogenesis (Ema and Hirose, 2006; Evans, 2018).

Lead and cadmium are ubiquitous heavy metals and have both been associated with testicular toxicity and impaired fertility in a number of species. Divalent lead is known to interact with physiological processes involving calcium and generally has an affinity for sulfhydryl groups (Evans, 2018). Lead is reported to be toxic to germ cells as well as Leydig cells and can suppress anterior pituitary secretion of LH and FSH (Evans, 2018; Thomas and Thomas, 2001). Lead also appears to be able to adversely affect the ability of spermatozoa to fertilize ova; however, this effect, like others associated with lead exposure, appears to be dependent on age and individual variations in susceptibility, adaptation, and reversibility (Evans, 2018; Sokol, 2006). Like lead, cadmium is thought to adversely affect male reproduction by inhibition of spermiation, as well as by interactions with the hypothalamic–pituitary–gonadal axis and adverse effects on the endothelium of the testicular and epididymal vasculature (Akinloye et al., 2006; Creasy and Foster, 2002; Evans, 2018; Thomas, 1995). Cadmium can also alter the junctional complexes between adjacent Sertoli cells and disrupt the integrity of the blood–testis barrier (Akinloye et al., 2006; Evans, 2018; Thomas and Thomas, 2001).

39.3.2.8.2 Adverse effects of heavy metals on female reproductive function

The ovaries do not appear to be as sensitive to the toxic effects of heavy metals as do the testes (Evans, 2018; Thomas, 1995). The neuroendocrine function of the hypothalamic–pituitary–gonadal axis appears to be targeted by lead in the female, as well as in the male (Evans, 2018). Anterior pituitary release of FSH and LH and ovarian steroidogenesis can be inhibited by cadmium (Evans, 2018; Hoyer, 2006; Thomas, 1995). Exposure of female laboratory animals to organotin compounds has been associated with reductions in follicular development and size, as well as the formation of corpora lutea, and it has recently been suggested that tributyltin and cadmium might also have estrogenic activities (Ema and Hirose, 2006; Evans, 2018; Golub, 2006b).

39.3.2.8.3 Adverse effects of heavy metals on embryonic/fetal development

Several heavy metals have been identified as teratogens and possible abortifacients in humans and animals, and the adverse effects of prenatal lead exposure on the developing nervous systems of both human and laboratory animal species have been well documented (Evans et al., 2003; Rice, 1998; Rogers, 2013). Prenatal exposure to organotins has been associated with pregnancy loss and impaired ossification in rodents (Ema and Hirose, 2006). The outcomes of an industrial accident in Japan and misuse of contaminated grain in Basra, Iraq, clearly demonstrated the developmental neurotoxicity of organic mercury (i.e., methyl mercury and related compounds) in humans and animals (Chang et al., 1998; Cordero, 1993; Golub, 2006b). Other heavy metals, including cadmium, have been associated with placental toxicity, as well as developmental neurotoxicity (Gupta, 2007; Hastings and Miller, 1998), and it has been recently reported that cadmium and other metals or metalloids might also have estrogenic effects (Golub, 2006b).

39.4 Conclusion

Reproduction is a critical biological process, required for financially viable livestock production, as well as long-term survival of human and animal populations. Toxicant-induced abortions, congenital defects, and infertility can have devastating effects on humans, domestic animals,

and wildlife species. As a result of the impact of these negative reproductive outcomes, there are growing global concerns about all of the documented potential adverse effects, including those on reproduction, associated with CWAs and other military- or terrorist-related chemical exposures. Adding to these legitimate concerns about the potential adverse health effects of various toxicants being used as weapons of war are the uncertainty, stress, and, panic which can be created by widely and almost instantaneously disseminated, weaponized disinformation. The facts presented in this chapter are intended to familiarize the reader with terminology and concepts pertinent to reproductive toxicity, including endocrine disruption, and to provide an overview of what is currently understood about the adverse reproductive effects of selected, potential CWAs. Additionally, readers need to be keenly aware of emerging, CWA-related issues involving cybersecurity failures and aggressive disinformation campaigns. It is hoped that the information and references provided in this chapter will assist readers in making informed decisions in their interpretation of experimental or epidemiological data concerning potential CWAs. Likewise, this chapter should assist readers in their preparation for future CWA experiments and/or clinical investigations, as well as attempts to weaponize patently false CWA-related information against specific human populations.

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Chapter 40

Liver toxicity of chemical warfare agents

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40.1 Introduction

Xenobiotic-induced liver injury has become the most frequent cause of acute liver failure in humans in the United States and around the world, exceeding all other causes combined (Watkins and Seef, 2006). Owing to its detoxification mechanisms, the liver protects the individual against xenobiotic-induced injury. Liver toxicity caused by chemical warfare agents (CWAs) is a potential area of concern.

Chemical-induced liver injury is encountered in a variety of circumstances. Some natural toxins, such as the peptides of *Amanita phalloides*, the pyrrolizidine alkaloids, the toxin of the cycad nut, and other plant toxins, are hazards posed by the environment. Some mycotoxins are ingested unknowingly because of feed contamination due to climatic conditions that favor fungal growth. Other circumstances of exposure to hepatotoxins include the contamination of a water supply with cyanobacterial toxins, which led to the tragic deaths of 60 patients in a hemodialysis clinic in Brazil in 1996 (Jochimsen et al., 1998).

Research in the last decade has focused on elucidating different mechanisms for chemical-induced liver injury. Investigators have attempted to understand the basis for such hepatic injury. The goal of this chapter is to provide a basic understanding of liver pathophysiology and to introduce the general concepts of liver injury. The chapter also describes some examples of CWAs that can inflict liver damage.

40.2 Structural organization of the liver

The hepatic lobule and hepatic acinus are relatively well-accepted models to describe the structure and functional aspects of the liver. Histologically, the *hepatic lobule* is a hexagonal region of the liver parenchyma around the central vein. Typically, six portal triads, consisting of branches from the portal vein and hepatic artery, as well as bile ductules, border the edge of the lobule. Cords of hepatocytes are arranged radially around the central vein, and blood sinusoids form between them. The hepatic parenchyma is divided into three zones based on proximity to the central vein. The area closest to the central vein is termed *centrilobular*, the area near the portal triads is *periportal*, and the area between the centrilobular and periportal parenchyma is termed *midzonal*.

Alternatively, *hepatic acinus* is defined as the structural and functional unit in the liver based on the hepatic microcirculation. In simple terms, the hepatic acinus is defined as a parenchymal mass organized around the portal triad. Within the acinus, blood drains from the portal area via the sinusoids into the central hepatic vein. The acinus is arbitrarily divided into zone 1, which corresponds to the periportal zone of the hepatic lobule; zone 2, which corresponds to the midzonal parenchyma; and zone 3, which corresponds to the centrilobular zone. The majority of blood supply to the liver is from the portal vein. Approximately 60%−80% of the blood originates from branches of the portal vein and supplies nutrients and toxins from the gastrointestinal tract, while 20%−40% of the blood originates from the hepatic artery, supplying oxygen (Treinen-Moslen and Klaasen, 2001). The blood from the portal vein and hepatic artery is mixed in the penetrating vessels, which then enter the sinusoids. Blood flows sequentially through zone 1, zone 2, and zone 3 before draining via the central vein. Because of this preferential blood circulation, hepatocytes in zone 1 receive blood that is 9%−13% oxygenated, whereas zone 3 is relatively hypoxic—the blood is 4%−5% oxygenated and nutrient-depleted (Treinen-Moslen and Klaasen, 2001).

In addition to hepatic parenchymal cells, which are hepatocytes, hepatocyte stem cells, termed *oval cells*, are reported to be located in the canals of Hering, where bile canaliculi from the hepatic cords converge on bile ductules of the portal triad. It is postulated that new hepatocytes travel down hepatic cords to replace the aging and

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damaged hepatocytes from zone 3. Sinusoids lined by specialized endothelium are blood channels located between hepatocyte cords. The endothelial lining of the sinusoids is discontinuous and has fenestrae to facilitate the movement of fluid and molecules less than 259 kDa in size (Watkins et al., 1999; Treinen-Moslen and Klaasen, 2001; Plumlee, 2004). This material enters the space of Disse, which is located between the endothelium and the hepatocytes. Within the space of Disse, hepatocytes contact free and protein-bound molecules, which may be absorbed by diffusion or active transport.

40.2.1 Hepatic functional capacity

The liver contributes to a plethora of functions. Liver filters the blood drained from the gastrointestinal tract via the portal vein for xenobiotics, endotoxins, ammonia, and other bacteria-derived products (Treinen-Moslen and Klaasen, 2001; Plumlee, 2004). The liver is directly involved in glucose homeostasis (Treinen-Moslen and Klaasen, 2001; Piñeiro-Carrero and Piñeiro, 2004); cholesterol synthesis and uptake (Treinen-Moslen and Klaasen, 2001); synthesis of proteins such as clotting factors, albumin, and very-low-density lipoprotein (Treinen-Moslen and Klaasen, 2001; Plumlee, 2004); storage of glycogen, lipids, minerals, and vitamins (Plumlee, 2004); metabolism and excretion of hemoglobin breakdown products (Plumlee, 2004); steroid hormones (Brown, 2001); and drug metabolites.

The hepatic zones have remarkable regiospecificity and metabolic diversity to accommodate the numerous functions of the hepatocytes. In addition to differences in oxygen gradients (zone 3 hepatocytes are oxygen-depleted compared to zone 1 hepatocytes), hepatocytes of zone 3 in particular are rich in drug-metabolizing enzymes. Zone 3 hepatocytes are involved in glycolysis and lipogenesis (Plumlee, 2004), and zone 1 hepatocytes are mitochondria-rich (Treinen-Moslen and Klaasen, 2001; Plumlee, 2004). Functions of zone 1 hepatocytes include bile salt extraction, fatty acid oxidation, gluconeogenesis, and protein synthesis (Treinen-Moslen and Klaasen, 2001; Piñeiro-Carrero and Piñeiro, 2004; Plumlee, 2004). Zone 1 hepatocytes have the highest levels of glutathione (GSH; Treinen-Moslen and Klaasen, 2001).

Bile secretion is a major function of the liver. Bile is composed of bile salts, bilirubin, GSH, phospholipids, cholesterol, proteins, organic anions, metals, and conjugated xenobiotics (Treinen-Moslen and Klaasen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Bile salts and bilirubin enter bile canaliculi via active transport through hepatocyte membranes. Canaliculi are dynamic structures located between hepatocytes and formed by hepatocyte membranes (Treinen-Moslen and Klaasen, 2001; Plumlee, 2004). Energy-dependent transport exists for certain hormones, drugs, and other xenobiotics. These include a group of multiple drug-resistance p-glycoproteins that transport lipophilic cationic drugs, estrogens, phospholipids, and canalicular multiple organic anion transporters involved in the movement of molecules conjugated to GSH, glucuronide, and sulfate. Metal and mineral transport is important for mineral homeostasis and occurs through facilitated diffusion and receptor-mediated endocytosis across the sinusoidal membrane. Lysosomes are involved in storage and export of metals and minerals into canaliculi.

Canaliculi enter canals of Hering in the portal triad and lead to intrahepatic bile ducts, which coalesce to form the hepatic bile duct. The bile duct empties the bile into the gallbladder, which then is released into the duodenum. Bile that is excreted into the small intestine enhances nutrient uptake, protects enterocytes from oxidation, and facilitates the excretion of xenobiotics and endogenous waste in the feces (Treinen-Moslen and Klaasen, 2001).

40.2.2 Hepatic cellular components

In addition to the hepatocytes and the hepatocytic stem cells (oval cells), which are parenchymal in origin, there are four types of nonparenchymal cells present within the liver. The nonparenchymal cells include the endothelial cells lining the sinusoids; bile duct epithelium; Kupffer cells, which are resident macrophages; the *stellate* cells, also called *Ito cells* or *fat-storing cells*; and the pit cells or large granular lymphocytes. In the rat, hepatocytes represent about 60% of the total cell number and 80% of hepatic tissue volume. Nonparenchymal cells in the rat are estimated to constitute about 30% of total cellular population, but comprise only 6%−7% of tissue volume due to their small size relative to hepatocytes (Dahm and Jones, 1996).

Kupffer cells represent 80% of the fixed macrophages in the body. These cells are mostly located within the sinusoidal lumina in close association with endothelial cells. Kupffer cells function as phagocytes, ingesting foreign material which may arrive through the portal circulation (Treinen-Moslen and Klaasen, 2001; Plumlee, 2004), as well as apoptotic or necrotic hepatocytes. Kupffer cells have other immune functions in that they act as antigen-presenting cells and secrete various cytokines. Kupffer cells may store minerals and are also involved in the pathogenesis of a variety of liver diseases induced by toxins such as ethanol (Laskin, 1990; Thurman et al., 1998).

Stellate cells are located within the sinusoids and store fat and vitamin A (Treinen-Moslen and Klaasen, 2001; Piñeiro-Carrero and Piñeiro, 2004; Plumlee, 2004). In the event of liver injury, stellate cells may become activated

to a myofibroblast-like phenotype (Plumlee, 2004; Maddrey, 2005). Activated stellate cells produce collagen and play a role in the pathogenesis of hepatic fibrosis. Pit cells are natural killer cells that have antineoplastic actions (Treinen-Moslen and Klaasen, 2001; Plumlee, 2004). They are also involved in granuloma formation (Plumlee, 2004).

40.3 Factors influencing hepatic toxicity

40.3.1 Preferential hepatic uptake

As mentioned previously, the liver has a dual blood supply. The hepatic artery delivers material from the systemic circulation and the portal blood flow delivers directly from the gastrointestinal system. The portal system is involved in the *first pass effect*, where the nutrients and xenobiotics that are absorbed from the stomach and intestines are filtered through the liver before reaching the systemic circulation (Treinen-Moslen and Klaasen, 2001). The space of Disse allows close contact between circulating plasma, plasma proteins, and hepatocytes, allowing rapid diffusion of lipophilic compounds across the hepatocyte membrane. Some compounds are specifically taken up by sinusoidal transporters, including phalloidin from several species of mushrooms in the genus *Amanita*, microcystin produced by the cyanobacteria *Microcystis aeruginosa*, and bile acids.

Liver cells have the potential to accumulate high levels of metals and vitamins, which can lead to toxic injury. Excessive vitamin A storage in stellate cells acutely leads to the activation and proliferation of these cells (Treinen-Moslen and Klaasen, 2001), while chronic high levels can lead to hepatic fibrosis and portal hypertension, precipitating increased fibrosis (Zimmerman et al., 1999; Piñeiro-Carrero and Piñeiro, 2004; Maddrey, 2005). The liver is also responsible for iron homeostasis. There is a receptor-mediated uptake of iron from the sinusoids and sequestration in storage proteins such as ferritin. High levels of iron cause lipid peroxidation of zone 1 hepatocytes (Treinen-Moslen and Klaasen, 2001). A common example of hepatic accumulation of metals is copper-mediated liver toxicity noted in certain breeds of dogs (e.g., Bedlington terriers, Dobermans, and Dalmatians), where copper is stored within lysosomes of hepatocytes, resulting in progressive accumulation of copper, resulting in liver necrosis (Rolfe and Twedt, 1995).

40.3.2 Xenobiotic metabolic bioactivation

Most xenobiotic agents absorbed by the small intestine are highly lipophilic. Renal excretion is the primary mechanism of xenobiotic removal, but kidney excretion of lipophilic compounds, which are frequently protein-bound in the circulation, is poor (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins et al., 1999). Such lipophilic compounds must be metabolized to increase their water solubility for excretion (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman et al., 1999). Microsomal enzymes within the liver add functional groups or conjugate xenobiotics to water-soluble molecules to facilitate excretion. While these reactions often function in the detoxification of compounds, there is significant potential for toxification (Zimmerman et al., 1999). Examples of phase I reactions include oxidation, reduction, and hydrolysis. Phase I enzymes, which are predominantly located in zone 3 of the hepatic lobule, may produce reactive metabolites.

Many hepatic enzymes are present in the smooth endoplasmic reticulum (ER) of the hepatocyte. When liver tissue is homogenized, the ER breaks down into small vesicles known as *microsomes*; thus, these enzymes are termed *microsomal enzymes*. As a rule, microsomal enzymes require oxygen and reduced nicotinamide adenine dinucleotide (NADPH) to function (Dahm and Jones, 1996; Brown, 2001). Most phase I enzymes contain heme, giving them a red coloration, and they absorb light at a wavelength of 450 nm. Most cytochrome P450s act as mixed-function oxidases. Genes for cytochrome P450s are highly conserved in mammals. There are three gene families, CYP1, CYP2, and CYP3, and more than 36 cytochrome P450 isoenzymes have been identified in animals (Dahm and Jones, 1996; Watkins et al., 1999).

Oxidation is the major phase I reaction produced by the group of cytochrome P450s. Important substrates for CYP450s include steroid hormones and lipid-soluble drugs (Brown, 2001). Oxidative reactions frequently lead to the formation of highly reactive epoxides. These toxic metabolites are usually detoxified rapidly by phase II conjugation or other mechanisms, such as microsomal epoxide hydrolases (Watkins et al., 1999; Piñeiro-Carrero and Piñeiro, 2004).

Noncytochrome P450 enzymes may also be involved in oxidative reactions. One such enzyme is alcohol dehydrogenase, whose substrates include vitamin A, ethanol, and ethylene glycol. Aldehyde dehydrogenase is another enzyme. Most reduction reactions also involve microsomal enzymes, with the exception of ketone reduction. Nitro compounds are reduced to amines, and volatile anesthetics undergo dehalogenation by microsomal enzymes. Hydrolysis reactions are involved in the metabolism of compounds with amide bonds or ester linkages, as in the conversion of aspirin to salicylate (Brown, 2001).

40.3.3 Phase II/conjugation reactions

Phase II enzymes may be cytosolic or microsomal (Dahm and Jones, 1996; Brown, 2001). Phase II enzymes are

predominantly involved in conjugating phase I metabolites or xenobiotics with functional groups. Phase II metabolites are rarely reactive, but there are a few exceptions, such as the glucuronide of the nonsteroidal antiinflammatory drug diclofenac and the GSH conjugate of α-naphthothiourea (ANTU). Phase II enzymes conjugate a polar group to the substrate at a hydroxyl group, carboxyl group, amino group, or sulfhydryl group produced through the actions of phase I microsomal enzymes. Polar molecules that are added to the substrate include glucuronic acid, sulfate derived from sulfuric acid ester, acetate, GSH, methyl groups derived from methionine, and amino acids such as glycine and cysteine. These polar groups significantly increase the water solubility of the substrate, facilitating rapid renal or biliary excretion.

Glucuronidation is the most common phase II reaction in humans, though it is deficient in the neonate (Sturgill and Lambert, 1997; Brown, 2001; Piñeiro-Carrero and Piñeiro, 2004). Substrates for glucuronidation usually include steroid hormones, thyroxin, and bilirubin, as well as many drugs, including salicylates and acetaminophen. Glucuronyl transferases are microsomal enzymes that catalyze the transfer of glucuronide from uridine 5′-diphosphate (UDP; Watkins et al., 1999). UDP may be depleted in patients overdosed with acetaminophen or other drugs that undergo this detoxification pathway. Products of glucuronidation may be excreted in the bile or urine. Those excreted in the bile may undergo hydrolysis in the intestine, which leads to reabsorption of the parent compound in a phenomenon called *enterohepatic cycling* (Brown, 2001). Similar to CYP 450 enzymes, some agents can also induce glucuronyl transferases, such as phenobarbital (Sturgill and Lambert, 1997).

Sulfation is the primary conjugation reaction for substrates with phenol groups or aliphatic alcohols (Sturgill and Lambert, 1997; Brown, 2001). These reactions are catalyzed by sulfotransferases in the cytoplasm. Agents that undergo sulfation include acetaminophen, morphine, ascorbic acid, and endogenous compounds like chondroitin, heparin, and some steroids. The pool of available sulfates may become saturated in drug overdoses.

Drugs with amine and hydrazine groups may be conjugated to acetate (Sturgill and Lambert, 1997). Sulfonamides often undergo acetylation (Brown, 2001). *N*-Acetyltransferase is an enzyme in the cytoplasm involved in acetylation reactions.

GSH and cysteine both have sulfhydryl groups that readily bind many phase I metabolites (Brown, 2001). GSH is a free-radical scavenger that prevents membrane damage from reactive metabolites. These reactions may be spontaneous or catalyzed by GSH peroxidases, which are selenium-dependent enzymes. Because these enzymes are cytosolic, damaged membrane phospholipids must be released by phospholipase A2 for detoxification. GSH is also involved in the reduction and recycling of other antioxidants, such as vitamins E and C (Dahm and Jones, 1996). When oxidized, GSH forms a dimer, which must be reduced by GSH reductases, which are NADPH-dependent enzymes. GSH may be depleted in the acetaminophen-overdosed or fasting person (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Piñeiro-Carrero and Piñeiro, 2004). *N*-Acetylcysteine is frequently used to replenish GSH.

40.3.4 Phase III reactions

In addition to phase I and II biotransformation enzymes, studies suggest the involvement of hepatic transporter systems involved in drug efflux from hepatocytes as a means for the liver to rid itself of foreign chemicals. These are termed *phase III transporter systems*. Several transporter families that mediate uptake of chemicals into liver and excretion of chemicals from liver into blood, bile, or both have been cloned and identified. In general, the organic anion transporting polypeptide family (Oatps), along with organic cation transporter 1 (Oct1) and organic anion transporter 2, mediate the uptake of a large number of xenobiotics from the blood to the liver. Conversely, multidrug resistance proteins (Mdrps), multidrug resistance-associated proteins (Mrps), and breast cancer resistance protein (Bcrps) mediate the efflux of xenobiotics from the liver into bile or blood (Klaassen and Slitt, 2005).

40.3.5 Pathologic manifestations of hepatic injury

40.3.5.1 Hepatic steatosis/fatty liver

Hepatic steatosis is the accumulation of fat droplets within the hepatocytes. Steatosis is usually a common response noted with a variety of liver toxicants and represents a potentially reversible injury to hepatocytes (Treinen-Moslen and Klaasen, 2001). Grossly, the affected liver will be swollen, with rounded edges, friable, and light brown to yellow in color. Compounds that produce prominent steatosis include the antiepileptic drug valproic acid and the antiviral agent fialuridine. Other toxins that may cause hepatic steatosis include aflatoxin and white or yellow phosphorus. Although steatosis has been considered benign and reversible, there are recent reports that suggest the progression of the steatosis stage to steatohepatitis, fibrosis, and cirrhosis (Ramaiah et al., 2004). Recently, there has been a syndrome noted in obese individuals, who are often type 2 diabetics, called nonalcoholic fatty liver disease (NAFLD), where hepatocytes are markedly steatotic and there is a marked inflammatory component (Diehl, 2005). Other disorders that result in fatty livers include hepatotoxic chemicals such

as thioacetamide, ethanol, and carbon tetrachloride. It should be noted, however, that several endocrine abnormalities result in steatosis, thus assigning the cause to a specific etiology should be done with caution.

Steatosis is termed *microvesicular* if the fat droplets are small and do not completely displace the nucleus. Microvesicular steatosis likely indicates a slow lipid accumulation (Bastianello et al., 1987; Plumlee, 2004) and may indicate a deficiency in mitochondrial β-oxidation of fatty acids. It is a relatively severe form of steatosis and has been associated with certain toxins, including aflatoxin in primates and dogs (Bastianello et al., 1987; Zimmerman et al., 1999) and valproic acid in humans (Sturgill and Lambert, 1997; Zimmerman et al., 1999). In contrast, macrovesicular steatosis describes hepatocytes containing large, usually single, fat droplets that displace the hepatocyte nucleus to the periphery of the cytoplasm. Macrovesicular steatosis indicates an imbalance between fatty acid uptake and secretion of very-low-density lipoproteins. This may be due to increased triglyceride mobilization, decreased fatty acid oxidation, decreased synthesis of very-low-density lipoproteins, or other metabolic anomalies (Sturgill and Lambert, 1997; Zimmerman et al., 1999; Treinen-Moslen and Klaasen, 2001; Plumlee, 2004).

40.3.5.2 Steatohepatitis

Steatohepatitis is the accumulation of lipids and the presence of inflammatory cells within hepatic parenchyma. Steatohepatitis is usually the next stage of steatosis if untreated (Lieber, 1994; Bautista, 2002; French, 2003). The inflammatory cells are usually neutrophils and macrophages. Conditions usually associated with steatohepatitis are alcoholic liver disease, NAFLD, and endotoxemia secondary to intestinal disease. Any toxic compounds that cause steatosis can also result in steatohepatitis if the condition is left untreated. Steatohepatitis may progress to fibrosis/cirrhosis and hepatocellular carcinoma if the inciting cause is not removed or treated (Diehl, 2002).

40.3.5.3 Apoptosis versus necrosis

Two forms of cell death are described within hepatocytes: apoptosis and necrosis. As with other organs, apoptosis is often called *programmed cell death* and is a normal physiologic process. Individual cells are affected (Dahm and Jones, 1996), cell death is not associated with inflammation, and normal architecture of the hepatic parenchyma is maintained, allowing regeneration (Treinen-Moslen and Klaasen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Apoptotic cells undergo cell shrinkage and nuclear condensation and pyknosis, but mitochondrial function (Piñeiro-Carrero and Piñeiro, 2004) is maintained and the cell membrane remains intact (Zimmerman et al., 1999). Apoptotic cells are occasionally seen in the centrilobular area but are rapidly phagocytosed by macrophages and other hepatocytes (Plumlee, 2004). Apoptosis may be induced by xenobiotics due to oxidative stress (Piñeiro-Carrero and Piñeiro, 2004), decrease in apoptotic suppressors, or enhanced expression of apoptosis genes (Dahm and Jones, 1996).

Necrosis is the predominant form of cell death in most hepatotoxic insults. The term *necrotic* is used to describe dead and dying cells, which are often identified by homogeneous eosinophilic cytoplasm on hematoxic and eosin-stained liver sections with variable loss of nuclear and cellular detail. Degenerative changes to the hepatocyte may precede necrosis. During necrosis, cells lose osmotic homeostasis and there is swelling of hepatocytes and organelles on an ultrastructural basis (Dahm and Jones, 1996; Treinen-Moslen and Klaasen, 2001), both of which can be observed microscopically. Energy production fails due to loss of calcium homeostasis (Dahm and Jones, 1996; Zimmerman et al., 1999). Eventually, the cell membrane ruptures, and leakage of cell contents occurs.

Necrosis is often initiated by damage to membranes, either the plasma membrane of the cell or the membranes of organelles, particularly mitochondria (Zimmerman et al., 1999). Cell membrane damage is often caused by membrane phospholipid peroxidation. Plasma membrane damage interferes with ion regulation, calcium homeostasis, energy production, and causes a decrease in the ability of that organelle to sequester calcium. Inhibition of protein synthesis is an alternative mechanism that may cause cell necrosis. Toxins that act in this way include phalloidin and related mushroom toxins, which inhibit the action of ribonucleic acid (RNA) polymerase, and therefore mRNA synthesis (Piñeiro-Carrero and Piñeiro, 2004).

40.3.5.4 Hepatic pigment accumulation

Various substances may accumulate within hepatocytes or Kupffer cells. These substances may be visible by microscopy as pigment. Occasionally, these pigments lend a grossly visible tint to the liver. Bile pigment may accumulate in canaliculi and bile ducts, particularly in zone 3, leading to a yellow to green color (Plumlee, 2004; Zimmerman et al., 1999). Iron, in the form of hemosiderin, is stored in the liver as a yellow-brown pigment that may be visualized using Pearl's Prussian blue. Copper may be yellow-brown and is visualized using rhodenase. Lipofuscin may be present within hepatocytes as a senile change. This yellow-brown pigment represents lipid accumulation within lysosomes.

40.3.5.5 Hepatic cholestasis

Cholestasis may be transient or chronic (Treinen-Moslen and Klaasen, 2001) and may be subdivided into

canalicular cholestasis and cholangiodestructive cholestasis. Canalicular cholestasis can be produced by drugs or other chemicals that damage the bile canalicular structure and function. A key component of bile secretion involves several adenosine triphosphate (ATP)-dependent export pumps, such as the canalicular bile salt transporter, that moves bile salts and other transporters that export bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. Some of the drugs bind these transporter molecules, resulting in the arrest of bile formation or movement within the lumen of the canalicular system (Klaassen and Slitt, 2005). Secondary bile injury can result if there is cholestasis due to the detergent action of bile salts on the biliary epithelium or hepatocytes in areas of cholestasis. Cholestasis can occur simply as a result of physical obstruction of canaliculi within the liver parenchyma (intrahepatic) or outside the liver (extrahepatic). Causes of cholestasis may include hepatobiliary tumors, endotoxemia, hepatocyte swelling, and intraductal crystals such as calcium salts of plant saponins. Disruption of actin filaments within the hepatocyte may cause cholestasis by preventing the normal pulsatile contractions that move bile through the canalicular system to the bile ducts. Drugs that bind to actin filaments such as phalloidin, those that affect cytoskeletal assembly such as microcystin, and those that affect calcium homeostasis and cellular energy production can generate this type of energy.

Cholangiodestructive cholestasis is caused by bile duct obstruction, which may be intrahepatic or extrahepatic. Bile duct injury may lead to the sloughing of epithelial cells into the lumen, cell edema, and inflammation, which may contribute to obstruction (Treinen-Moslen and Klaasen, 2001; Plumlee, 2004). Chronic lesions associated with cholangiodestructive cholestasis typically include bile duct proliferation and periductular fibrosis. Vanishing bile duct syndrome, characterized by a loss of bile ducts, has been seen in chronic cholestatic disease in humans (Zimmerman et al., 1999; Treinen-Moslen and Klaasen, 2001) and has been produced experimentally in dogs (Uchida et al., 1989).

40.3.5.6 Hepatic fibrosis

Fibrosis usually results from chronic inflammation, which can be the result of continuous exposure to a variety of hepatotoxic chemicals, such as organic arsenicals, vinyl chloride, or high doses of vitamin A (Zimmerman et al., 1999), chronic ethanol ingestion, and NAFLD. Fibrosis usually occurs around the portal area, in the space of Disse, and around the central veins. This results in loss of liver architecture and function. The hepatocytes are replaced with fibrous material, and thus there is hepatocyte loss. Periportal fibrosis may lead to portal hypertension.

40.3.5.7 Cirrhosis

Hepatic cirrhosis is typically the end stage of liver disease. Cirrhosis describes an irreversible change (Treinen-Moslen and Klaasen, 2001) characterized by accumulation of excessive collagen deposition in the form of bridging fibrosis, which disrupts the hepatic architecture. Cirrhosis may be micronodular or macronodular, depending on the amount of fibrosis and tissue regeneration. Liver transplantation is the only solution to restore adequate liver function in human medicine.

40.3.5.8 Pathomechanisms of hepatic injury

Mechanisms of liver injury have been divided into two categories: intrinsic and idiosyncratic. Intrinsic injury may lead to steatosis, necrosis, cholestasis, or a mixed form of damage, often with minimal inflammation (Sturgill and Lambert, 1997). Intrinsic liver injury is a predictable, reproducible, dose-dependent reaction to a toxicant (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman et al., 1999; Piñeiro-Carrero and Piñeiro, 2004). A threshold dose exists for xenobiotics causing intrinsic liver injury. There is commonly a predictable latent period between the time of exposure and clinical evidence of liver injury. This type of liver injury accounts for the vast majority of toxic liver injury and is often caused by reactive products of xenobiotic metabolism, most commonly electrophiles and free radicals. A few drugs cause intrinsic liver injury without bioactivation. An abbreviated summary of mechanisms of intrinsic liver injury is illustrated in Fig. 40.1.

Idiosyncratic responses are, by contrast, unpredictable responses to a drug or other toxicant. They are rare, non-dose-dependent, and often associated with extrahepatic changes (Sturgill and Lambert, 1997; Zimmerman et al., 1999; Piñeiro-Carrero and Piñeiro, 2004; Shenton et al., 2004). Idiosyncratic drug reactions often occur after sensitization, followed by reexposure to a drug. There is a usually a delay of 1−5 weeks (and occasionally several months) between the time of the first dosing and the time that clinical signs become evident, but onset is expedited with rechallenge (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins et al., 1999). Hepatic changes associated with idiosyncratic drug reactions include necrosis, cholestasis, or both; and there is often an inflammatory response involving macrophages and eosinophils. Extrahepatic clinical signs may include pyrexia, rash, and peripheral eosinophilia. Some idiosyncratic drug reactions resemble serum sickness. Some of the mechanisms of liver injury are described in more detail next.

40.3.6 Oxidative stress and free radicals with classic examples

Free radicals are generated within hepatocytes in several ways, such as ionizing radiation, oxidative metabolism by

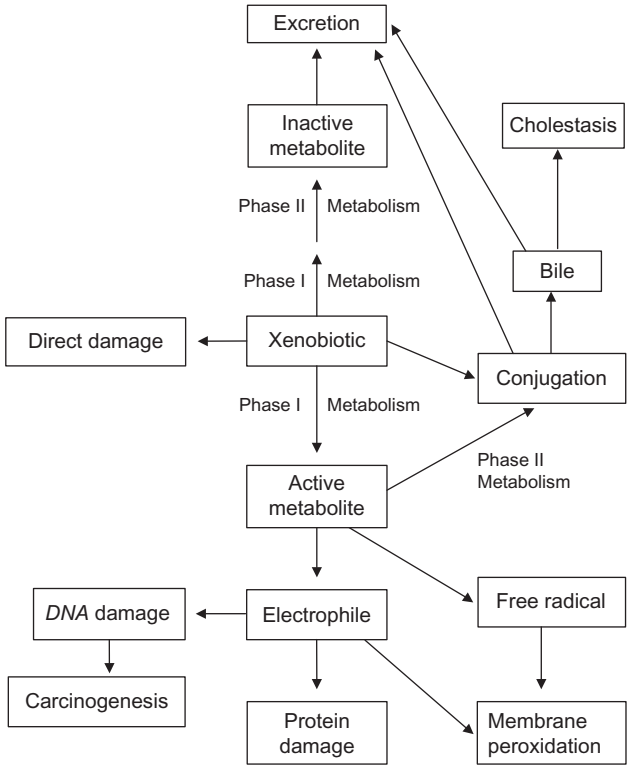

FIGURE 40.1 Multiple metabolic pathways involved in the mediation of hepatic injury for any compound. The liver is central to the metabolism of xenobiotic (and some endogenous) compounds, which produces water-soluble products amenable to urinary or biliary excretion. Some compounds undergo metabolic activation to produce free radicals, electrophiles, or other toxic products that may induce hepatic injury. *Adapted from Gupta, R. (Ed.), 2007. Veterinary Toxicology, first ed. Academic Press, p. 158.*

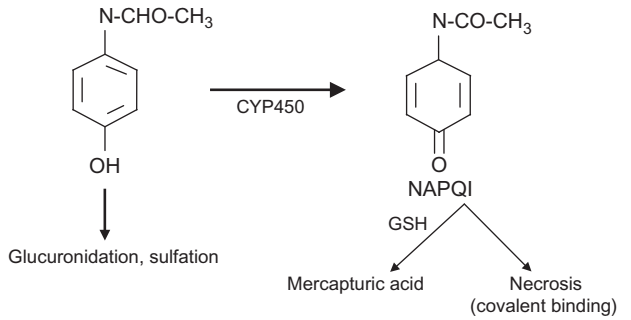

FIGURE 40.2 Metabolism and mechanism of acetaminophen toxicity. Bioactivation of acetaminophen by P450 enzymes results in the formation of the reactive intermediate (NAPQI), which forms covalent protein adducts with GSH, which then is converted to mercapturic acid. When the amount of the reactive metabolite formed exceeds the GSH available for binding, the excess metabolite binds to tissue molecules, resulting in centrilobular hepatic necrosis. *Adapted from Gupta, R. (Ed.), 2007. Veterinary Toxicology, first ed. Academic Press, p. 155.*

cytochrome P450, reduction and oxidation (redox) reactions that occur during normal metabolism, transition metals such as iron and copper, and from nitric oxide generated by a variety of inflammatory cells. The reactive species that are generated result in lipid peroxidation of membranes, oxidative modification of proteins, and lesions within DNA (Crawford, 1999).

Free radicals have unpaired electrons, making them highly reactive. They may be formed by one electron oxidation or reduction reaction, leading to cationic or anionic radicals, respectively (Dahm and Jones, 1996). Alternatively, hemolytic bond scission leads to neutral radical formation. Oxygen free radicals result from metabolic processes, leukocytic respiratory burst, or the effects of ionizing radiation. Hydroxyl radicals, superoxide radicals, and hydrogen peroxide are major reactive oxygen species. The free radical nitric oxide (NO), an important cell-signaling agent released by leukocytes, may react with superoxides to form peroxynitrite.

Free radicals cause peroxidation of phospholipids within the plasma membrane of the cell, as well as the membranes of the mitochondria and ER. The radicals act by removing a proton (H^+) from a methylene carbon within a polyunsaturated fatty acid, forming a lipid free radical. This lipid free radical then may abstract a proton from a neighboring polyunsaturated fatty acid, generating more lipid free radicals. It is estimated that this can occur 4–10 times per initiation. The effects of lipid peroxy radicals on the cell membrane include increased permeability, decreased fluidity, and inactivation of membrane proteins (Dahm and Jones, 1996). Additionally, mitochondrial membranes lose polarity (Watkins et al., 1999).

Lipid peroxy radicals can react with metal ions stored within the hepatocyte, generating more lipid radicals. It is estimated that propagation by this mechanism can occur in 4–10 steps per initiation (Dahm and Jones, 1996). The most frequent mechanism of free-radical production leading to hepatocellular injury involves phase I metabolism of xenobiotics and the cytochrome P450 system. Phase I metabolism may lead to bioactivation of the substrate to a high-energy reactive intermediate molecule in preparation for phase II conjugation reactions. However, in circumstances such as overdosage, phase I products may accumulate. Lesions produced by these compounds are mostly centrilobular because the cytochrome P450s responsible for metabolism are mostly situated in the centrilobular areas of the liver.

The classic examples of this process are cell death resulting from carbon tetrachloride and acetaminophen toxicosis (Fig. 40.2). Acetaminophen has a hydroxyl group that can undergo immediate phase II conjugation reactions. Indeed, at therapeutic doses, 90% of this substrate undergoes glucuronide or sulfate conjugation in humans (Court and Greenblatt, 1997; Sturgill and Lambert, 1997). These are major metabolic pathways in most species, but the glucuronyl transferase deficiency in cats in part explains the sensitivity of felines to this drug.

Acetaminophen in itself is not considered toxic. Cellular injury is caused by the unstable metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI; Fig. 40.2). Under normal conditions in humans, 5% of a dose of acetaminophen is oxidized to NAPQI, which is rapidly neutralized by conjugation with GSH (Sturgill and Lambert, 1997; Maddrey, 2005; Fig. 40.2). Toxic levels of NAPQI may accumulate under certain conditions, as when large amounts of substrate are available for metabolism due to either large ingestions or inadequate glucuronidation.

Metabolism of acetaminophen to NAPQI is increased in individuals who regularly consume alcohol or take medications that induce microsomal enzymes (e.g., antiepileptic therapy). The hepatic pool of GSH becomes overwhelmed and depleted, permitting the accumulation of NAPQI. Possible additional risk factors that lower the threshold for hepatotoxicity have been identified; they include fasting and malnutrition, which deplete GSH reserves (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Treinen-Moslen and Klaasen, 2001; Piñeiro-Carrero and Piñeiro, 2004).

The reaction that produces NAPQI generates superoxide anions as a by-product. Interactions of NAPQI with other cellular molecules also generate reactive oxygen species, leading to oxidative stress on hepatocytes (Dahm and Jones, 1996; Zimmerman et al., 1999). The roles of calcium and Kupffer cell activation have been implicated as contributing factors for acetaminophen-induced liver injury by producing reactive nitrogen species (Treinen-Moslen and Klaasen, 2001).

NAPQI also acts as an electrophile, targeting the mitochondria in particular. This reactive metabolite forms covalent adducts with cellular molecules, particularly proteins with thiol groups. Other targets in addition to mitochondrial proteins include plasma membrane proteins involved in calcium homeostasis and adenine nucleotides (Dahm and Jones, 1996; Sturgill and Lambert, 1997).

40.3.7 Disruption of calcium homeostasis

Calcium ions (Ca^{2+}) are important for the mediation of hepatic injury. Cytosolic-free calcium is maintained at relatively low concentrations compared to the extracellular levels. The majority of intracellular calcium is sequestered within the mitochondria and ER. Membrane-associated calcium and magnesium ATPases are responsible for maintaining the calcium gradient (Farrell et al., 1990). Significant and persistent increases in the intracellular calcium result from nonspecific increases in permeability of the plasma membrane, mitochondrial membranes, and membranes of the smooth ER. Calcium pumps in the mitochondrial membrane require NADPH; thus, depletion of available NADPH can cause the release of calcium from mitochondria (Cullen, 2005).

Elevated cytoplasmic calcium activates a variety of enzymes, with membrane-damaging effects. The major enzymes that are involved in activation by calcium include ATPases, phospholipases, proteases, and endonucleases. Thus, increased calcium causes increased mitochondrial permeability and induction of apoptosis and necrosis. Calcium is required for maintenance and function of the cytoskeleton as well (Dahm and Jones, 1996; Delgado-Coello et al., 2006).

Although cell injury results in increased calcium, which causes a variety of damaging effects, the cause-and-effect relationship of calcium to cell damage is not known. The chemicals that cause liver damage by this mechanism include quinines, peroxides, acetaminophen, iron, and cadmium.

40.3.8 Inhibition of mitochondrial function

Mitochondria function in the process of producing energy, in the form of ATP, for the cell by oxidative phosphorylation. Hepatocytes are highly metabolically active and require a continuous supply of ATP. Hepatocytes active in detoxification or replacement of damaged tissue have even greater ATP requirements (Dahm and Jones, 1996). Compounds that may disrupt mitochondrial oxidative phosphorylation include bile acids and amiodarone.

Mitochondria are also critical to modulation of cell redox status, osmotic regulation, pH control, and cytosolic calcium homeostasis and cell signaling. Mitochondria are important targets for virtually all types of injurious stimuli, including hypoxia and toxins. Mitochondria are targeted by oxidants, electrophiles, lipophilic cations, and weak acids. Damage is often precipitated by increases in cytosolic calcium.

Hepatic injury is frequently accompanied by morphological changes in mitochondria. Mitochondrial changes evident as structural abnormalities include greatly increased size and the development of crystalline inclusions. These changes are usually regarded as pathologic, reflecting either a protective or degenerative response to injury. Mitochondrial damage may result in the formation of high-conductance channels (the so-called mitochondrial permeability transition) in the inner mitochondrial membrane. This is an irreversible change, and because membrane potential is critical for mitochondrial oxidative phosphorylation, it constitutes a death blow to the cell.

Oxidative phosphorylation produces reactive oxygen species (Watkins et al., 1999). These are deactivated by antioxidants present within the mitochondrion. GSH is present within mitochondria as a scavenger for peroxides and electrophiles. Synthesis of GSH requires ATP and takes place outside the mitochondrion. A greater than 90% depletion in GSH reserves decreases the ability of the mitochondrion to detoxify reactive oxygen species

produced by oxidative phosphorylation. GSH *S*-transferase, the enzyme required for recycling of GSH, may become overwhelmed by toxicants and reactive metabolites (Dahm and Jones, 1996).

Xenobiotics may cause cell death by their effects on mitochondrial DNA. Some antiviral dideoxynucleoside analogs can disrupt mitochondrial DNA synthesis through the inhibition of DNA polymerase gammas, leading to depletion of mitochondria and consequent hepatocyte death.

Chemicals that damage mitochondrial structure, enzymes, or DNA synthesis can disrupt beta oxidation of lipids and oxidative energy production within hepatocytes. Prolonged interruption of beta oxidation leads to microvesicular steatosis, which can progress to macrovesicular steatosis. This sequence of events has been noted with both alcoholic and nonalcoholic steatohepatitis. The role of mitochondria has been extensively studied with NAFLD, a major issue in human medicine. Alcoholic steatosis and other forms of hepatic steatosis have been linked to impairment of ATP homeostasis, and mitochondrial abnormalities have been reported in a growing body of literature.

There are several drugs that inhibit beta oxidation of fatty acids in mitochondria leading to lipid accumulation, such as aspirin, valproic acid, and tetracyclines.

40.3.9 Autophagy and endoplasmic reticulum stress

Autophagy is a catabolic process for maintaining cellular homeostasis and integrity of organelles by eliminating cytosolic materials through vacuole-mediated sequestration and subsequent delivery to lysosomes for degradation. Autophagy has emerged as playing a critical role in the regulation of liver physiology and metabolism. During induction of autophagy, cytosolic components are encapsulated in a phagophore, which forms a double-membrane-bound vesicle termed an autophagosome. This newly formed autophagosome fuses with a lysosome, allowing degradation of encapsulated products, whereby molecules and proteins may be recycled to improve cell survival. Further, numerous studies indicate that autophagy plays a role in the pathogenesis of various liver disorders (Cursio et al., 2015). In diabetic mice, autophagy has been closely associated with ER stress during the development of steatohepatitis (Zhang et al., 2015). The expression of ER stress-signaling proteins like CCAAT/enhancer binding protein (C/EBP)-homologous protein (CHOP; a proapoptotic transcription factor) is positively associated with autophagic activity in liver tissues (Zhang et al., 2015). In the literature, ER stress has been indicated as a potent trigger for induction of autophagy (Rashid et al., 2015; Lee et al., 2015), it is possible that acute liver injury may lead to ER stress, which subsequently might activate autophagy.

40.3.10 Disruption of the cytoskeleton

Changes in intracellular calcium homeostasis produced by active metabolites of xenobiotics may cause disruption of the dynamic cytoskeleton. There are a few toxins that cause disruption of the cytoskeleton through mechanisms independent of biotransformation. Microcystin is one of these toxins. Microcystin is produced by the cyanobacterium *M. aeruginosa*. Similar toxins are produced by other species of cyanobacteria. The hepatocyte is the specific target of microcystin, which enters the cell through a bile-acid transporter. Microcystin covalently binds to serine/threonine protein phosphatase, leading to the hyperphosphorylation of cytoskeletal proteins and deformation of the cytoskeleton (Treinen-Moslen and Klaasen, 2001).

Phalloidin and related toxins found in some mushrooms, including *A. phalloides*, act by binding tightly to actin filaments and preventing cytoskeletal disassembly (Treinen-Moslen and Klaasen, 2001).

40.3.10.1 Cholestatic mechanisms

Sinusoidal transporters and canalicular transporters are involved in the movement of bile salts from the sinusoids into the canaliculi. Within the hepatocyte, transcytosis is mediated by cytoskeletal transport mechanisms. Bile is moved within the canaliculi through actions of the hepatocyte cytoskeleton, causing contraction of the canalicular lumina (Treinen-Moslen and Klaasen, 2001). Xenobiotics acting on any of the above systems may influence bile transport and secretion.

Most chemicals that cause cholestasis are excreted in the bile, including the mycotoxin sporodesmin, which concentrates 100-fold in the bile (Treinen-Moslen and Klaasen, 2001). α-Naphthylisothiocyanate (ANIT) is a hepatotoxicant that damages bile duct epithelium and hepatocytes. The drug is used experimentally in rodents as a model of intrahepatic cholestasis. A single dose of ANIT induces acute cholangitis; prolonged exposure causes bile duct hyperplasia and biliary fibrosis. Although the biochemical and histological features of ANIT toxicity are well documented, the mechanism by which ANIT causes liver injury remains uncertain. ANIT does not cause liver damage in vivo until it appears in bile (Jean and Roth, 1995).

This drug is initially detoxified in hepatocytes by conjugation with GSH. ANIT−GSH complexes are secreted into bile, but they are unstable and rapidly dissociate, which exposes biliary cells to high concentrations of the parent compound, which presumably causes direct

cytotoxicity. The reappearance of ANIT in bile also leads to enterohepatic cycling, reuptake of the drugs in the intestine, and repetitive rounds of GSH conjugation and secretion. This not only delays elimination of the drug, but also depletes GSH progressively from hepatocytes and leads to hepatocellular damage. In addition, ANIT is known to cause hepatotoxicity by neutrophil- and platelet-dependent mechanisms (Jean and Roth, 1995).

40.3.10.2 Idiosyncratic reactions

Many idiosyncratic drug reactions are believed to be immune-mediated. Neoantigens may result from adducts formed from the interaction of reactive drug metabolites with cellular proteins. These neoantigens may be processed by Kupffer cells or other antigen-presenting cells, transported to the cell surface, and presented as antigens. A cell- and antibody-mediated immune response may cause severe liver damage. Various drugs are believed to cause immune-mediated idiosyncratic reactions in humans, including halothane, diclofenac, phenytoin, and sulfonamides (Sturgill and Lambert, 1997; Watkins et al., 1999; Zimmerman et al., 1999; Treinen-Moslen and Klaasen, 2001).

Liver injury can be a result of both direct cytotoxicity and antibody-dependent cellular toxicity. Alcoholic liver disease is another example of possible immune-mediated damage. Acetaldehyde, produced by the metabolism of ethanol, forms adducts with hepatic proteins similar to halothane, resulting in higher antibody titers, to which some of the liver damage following ethanol ingestion may be attributed (Ramaiah et al., 2004). However, the role of immune-mediated liver damage following ethanol ingestion is minimal compared to other known mechanisms of alcohol liver damage.

40.4 Biological toxins

Toxins are biomolecules produced by living organisms mainly for defensive purposes. These molecules induce detrimental effects in other organisms, by different routes of exposure (Dorner and Rummel, 2015). Some of these toxins are linked to natural intoxications and are extremely dangerous due to their lethal doses (LD). An $LD_{50} < 25$ mg/kg indicates a very toxic substance, while substances having $LD_{50} > 2000$ mg/kg are not classified as toxic (Malmfors and Teiling, 1983).

Biological agents used for warfare are classified by the Centers for Disease Control and Prevention (CDC) into three categories. Category A consist of factors characterized by high pathogenicity and mortality as well as ease of dissemination (e.g., *Bacillus anthracis*). Category B pathogens are easy to spread, with low morbidity and mortality rates, but require specific monitoring and improved diagnostic capabilities (for example, ricin, abrin). Category C includes emerging pathogens against which the population has little or no immunity, and which could be engineered as a bioweapon (e.g., hantavirus) (Jansen et al., 2014).

40.5 Warfare agents affecting the liver

40.5.1 Fungal and plant toxins

40.5.1.1 Microcystins

The microcystins are hepatotoxic products of freshwater blooms of cyanobacteria of *Microcystis*, *Anabena*, and *Oscillatoria* species (Chen et al., 1993; Luu et al., 1993), with *M. aeruginosa* being the most common. Nearly 60 microcystin cyclic hepatopeptides have been identified, of which microcystin-LR, also known as the *fast death factor*, is the most common, and the toxin of choice to be weaponized (Craig et al., 1993; Rinehart et al., 1994). This toxin has been reported to be responsible for the deaths of wild animals and agricultural livestock (Carmichael, 1988). A potential threat to the health of humans has been recognized in countries where water supplies are contaminated with cyanobacteria (Yu et al., 1989; Gkelis and Zaoutsos, 2014). In 1996, microcystin-LR was also implicated in the death of 50 Brazilian dialysis patients (Jochimsen et al., 1998).

This potent mammalian liver toxin (Carmichael, 1988) acts by altering the hepatocyte cytoskeleton actin filaments, leading to disruption of the structural integrity of the sinusoids. This causes massive necrosis in the liver leading to cell death. The toxic effects of microcystin-LR have been reported due to the presence of 3-amino-9-methoxy-10-phenyl-2,6,8 trimethyl deca,4,6 dienoic acid (ADDA), which is believed to be essential for its functioning. In addition, microcystins are known to be potent and specific inhibitors of catalytic subunits of protein phosphatases 1 and 2A (Cohen and Cohen, 1989; Honkanen et al., 1990; Yoshizawa et al., 1991), and activate the enzyme phosphorylase b. Microcystin administered intraperitoneally in mice caused disruption of bile flow in less than 10 min, and death within a few hours due to hypovolemic shock induced by interstitial hemorrhage following liver necrosis (Carmichael, 1988). Death was also reported within hours of administration of aerosol (LD_{50}, 67 μg/kg body weight) in mice. In humans, microcystin ingestion leads to diarrhea, vomiting, weakness, and pallor, with death occurring in a few hours if a LD is taken.

40.5.1.2 Aflatoxins

Aflatoxins were first isolated more than 40 years ago after the outbreak of disease and deaths in turkeys.

These toxins are produced as secondary metabolites by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* at temperatures between 24°C and 35°C and humidity exceeding 7% (Williams et al., 2004). These fungi are known to infect a variety of crops, such as peanuts, walnuts, pecans, pistachios, and corn.

Aflatoxins are bisfuran polycyclic compounds, and depending on the characteristic blue or green fluorescence produced under ultraviolet light, these compounds are known as aflatoxin B_1, B_2, G_1, and G_2, respectively. Although these compounds have been recognized as potent mutagens and carcinogens, they are still not as toxic as botulinum or ricin. Before the first Gulf War, aflatoxins were not recognized as biological warfare agents; however, they were weaponized by Iraq for missile delivery, and at present, they can be considered as agents of biological warfare (Marshall, 1997; Zilinskas, 1997).

Of the several aflatoxins, aflatoxin B_1 (AFB_1) is the most prevalent and the most potent. AFB_1 is converted to an unstable metabolite (the 8,9-epoxide), is highly electrophilic, and forms covalent adducts with RNA, DNA, and proteins (Roebuck and Maxuitenko, 1994). In addition, aflatoxin M_1, a metabolite of AFB_1, has been found in the milk of cows fed AFB_1.

AFB_1 is acutely toxic in all species tested thus far, with an LD_{50} ranging from 0.5 mg/kg for the duckling to 60 mg/kg for the mouse (Wogan and Bushch, 1973). Acute exposure to AFB_1 has been reported to cause hepatic lesions with edema, biliary proliferation, and parenchymal cell necrosis. In addition, aflatoxin B_1 poisoning has been reported to cause jaundice, rapidly developing ascites, and portal hypertension, with high mortality resulting from massive gastrointestinal bleeding. In children, aflatoxin produces a condition called *Reye syndrome*, resulting in disturbed consciousness, fever, convulsions, and vomiting (Palmgren and Ciegler, 1983).

40.5.1.3 Ricin

Ricin, a potent plant toxin, was discovered by Peter Hermann Stillmark in 1889 (Flexner, 1987) as the first plant lectin. It was extracted from the seeds of castor plants (*Ricinus communis*) and has been considered a CWA since World War I. Although ricin is less toxic than botulinum or anthrax, easy availability and lack of a specific antidote make it a weapon of choice, and it has been included in Schedule 1 of the Chemical Weapons Convention (CWC). To date, more than 750 cases of intoxication in humans have been reported (Rauber and Heard, 1985). Being a ribosome-inactivating protein, a single molecule of ricin reaching the cytosol can kill that cell as a consequence of protein synthesis inhibition (Eiklid et al., 1980). Ricin is also very stable and extremely toxic by several routes of exposure, including ingestion and inhalation; however, compared to oral exposure, inhalation of ricin has been reported to be more harmful. In humans the LD has been reported to be 5−10 μg/kg body weight. Animal studies reveal that ricin is absorbed by the lymphatic and blood vessels with 2 h of ingestion, followed by accumulation in the liver and the spleen.

In addition to pulmonary toxicity, ricin has been reported to be a major hepatotoxicant (Kumar et al., 2003). Studies by Muldoon et al. (1992) reported that humans exposed to ricin experience increased hepatic transaminase and lactate dehydrogenase activities. Furthermore, mice treated with ricin experienced significant oxidative stress, resulting in hepatic GSH depletion and lipid peroxidation (Muldoon and Stohs, 1991). Elevation of lipid peroxidation is reported to originate mainly from the damaged Kupffer cells, which are believed to be the target of this toxin in the liver (Skilleter et al., 1981). The high sensitivity of Kupffer cells has been ascribed to the ability of ricin to bind to the mannose receptors present on these cells (Skilleter et al., 1981; Magnusson and Berg, 1993).

40.5.1.4 Abrin

Abrin, a potent toxin, is extracted from the seeds of the rosary pea (*Abrus precatorius*). Due to its easy availability and preparation, this toxin is an attractive option for weaponizing in poor countries, and thus has also been included in Schedule 1 of the CWC. The mechanism of action of abrin is very similar to that of ricin; however, in mice, abrin is 75 times more toxic than ricin (0.04 μg/kg for abrin is equivalent to 3 μg/kg of ricin). Similar to ricin, inhalation of abrin is found to be more toxic than ingestion. However, abrin ingestion has reported to be toxic to the liver, unlike ricin. At the cellular level, abrin is a potent toxalbumin known to cause cell death by inhibiting protein synthesis (namely type 2 ribosomal inhibitory protein). Further, abrin is also known to induce endothelial cell damage, leading to an increase in cell permeability, fluid and protein leakage, and tissue edema.

40.5.2 Bacterial (anthrax)

Anthrax is a disease caused by the spores of the bacterium *B. anthracis*. It is a recognized biological warfare agent. In the United States, anthrax was deliberately spread through the postal system in 2001 via letters sent to several legislators and other public figures with powder containing anthrax. This resulted in 22 cases of anthrax infection. Inhalation and cutaneous exposure are the main routes of infection. Recent studies have revealed that laboratory animals infected with anthrax have developed

significant liver injury. Studies by Moayeri et al. (2003) reported that mice infected with *B. anthracis* developed extensive liver necrosis and pleural edema. Liver lesions in these animals ranged from small hemorrhagic infarcts to large areas of centrilobular coagulative necrosis. A significant increase in alanine aminotransferase and aspartate aminotransferase was also observed in these animals. Liver dysfunction was also indicated by decreased serum albumin levels in these animals in response to toxin exposure over time.

40.6 Concluding remarks and future directions

In spite of significant advances in human knowledge about the mechanisms of liver damage, scientists seem to have a long way to go before all the mechanisms of toxicity in liver for a given chemical are completely established. However, with the advances in the technologies and techniques, especially the "omics approaches," researchers have been provided with additional tools to obtain breakthroughs in the area of liver toxicity. Clearly, current reports on the number of CWAs targeting the liver are limited, and with further research and case studies, these numbers will continue to grow. With the changing political global climate and the potential for CWAs, target organ toxicities, especially the liver, will likely gain added attention.

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Chapter 41

Renal system

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41.1 Introduction

The kidney and urinary system are responsible for the maintenance of homeostasis by regulating the body's fluid and electrolyte balance, eliminating waste products, maintaining acid−base balance, secreting regulatory peptides and hormones, and metabolizing and excreting endogenous compounds and xenobiotics. The kidney receives 20%−25% of cardiac output, putting it at risk for exposure to high levels of bloodborne toxicants. Besides direct exposure to toxic compounds, the kidney can be exposed to toxicants through metabolic activation of xenobiotics by renal tubular epithelial enzyme systems (e.g., cytochrome P450 enzymes). In its excretory capacity, the kidney and urinary tract are exposed to progressively higher levels of toxicants because they are concentrated within the renal/urinary tubules. Loss of renal function because of toxic kidney injury can result in severe systemic derangement and death of the individual; therefore, in theory, toxicants that target the kidney might be considered to be potential agents of chemical or biological warfare. However, if the goal of a chemical warfare agent is to rapidly incapacitate or kill civilians or opposing personnel, then a primary nephrotoxicant would make a poor choice of weapon because of the long delay in clinical effects from the time of exposure. Once renal damage has occurred, it may take many hours or up to a few days before signs of renal insufficiency develop to the point at which the victim is effectively incapacitated. Renal injury occurring subsequent to a chemical or biological attack is generally a matter of collateral damage rather than targeted injury, and many of the chemical agents used in warfare and terrorism have minimal effects on the kidney or urinary system. The renal effects that do develop will be seen in the individuals who survive the acute chemical attack and live long enough for evidence of renal insufficiency to develop.

41.2 Anatomy and physiology

41.2.1 Functional anatomy

In mammals, the kidneys are paired organs that reside ventrolateral to the lumbar vertebrae and musculature. Mammalian kidneys are bean-shaped to horseshoe-shaped with uniform to multilobulated external surfaces, depending on species, and a medial indented hilar region from which the renal artery, renal vein, lymphatics, nerves, and ureter emerge. On sagittal section, the kidney displays two distinct regions, the outer cortex and the inner medulla. The renal cortex corresponds to approximately 80% of total renal mass, and the normal cortex:medulla ratio is 1:2 to 1:3 in most species (Maxie and Newman, 2007). The medulla is divided into ray-shaped sections known as renal pyramids that have their bases at the corticomedullary junction and their apices (papillae) that empty into a renal calyx or pelvis at the hilar area. The number of renal pyramids in the kidney varies with species. Unipyramidal or unipapillate kidneys have a single renal papilla into which renal lobes empty, whereas multipyramidal or multipapillate kidneys have two or more papillae. From the hilar region, urine is channeled to the distal urinary bladder via the ureter, and from the bladder, urine is voided from the body via the ureter.

Renal blood flow originates from the renal arteries, which are direct branches off the aorta. Renal arteries progressively branch to form interlobar arteries, arcuate arteries, interlobular arteries, and afferent arterioles, the latter of which provide blood to the glomerulus. The kidney receives up to 20%−25% of cardiac output, with the cortex receiving the majority (90%) of the blood flow, and the medulla (6%−10%) and papilla (1%−2%) receiving considerably less direct blood flow (Schnellmann and Klaassen, 2008). Thus, bloodborne toxicants are delivered in higher amounts to the cortex, whereas the medulla and papilla are exposed to higher luminal levels of toxicants

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that concentrate in the urine. Because of the relative sluggishness of medullary and papillary filtrate transport, these areas are also exposed to intraluminal toxicants for prolonged periods of time.

The functional unit of the kidney is the nephron, which comprises the renal corpuscle (Bowman's capsule and the glomerulus), proximal tubule, loop of Henle, and distal tubule (Fig. 41.1). The number of nephrons per kidney varies with species size, ranging from approximately 10,000 in mice compared with approximately 1,000,000 in humans and 7,000,000 in elephants (Braun et al., 2008). High hydrostatic pressure from afferent arterioles results in ultrafiltration of plasma within the branching and anastomosing capillaries that form the glomerulus. Six- to nine-nanometer fenestrae within the glomerular basement membrane (GBM) form the "sieve" through which the plasma filtrate passes. Filtrate moves through Bowman's capsule and flows into the proximal renal tubule, which has three anatomically and functionally different segments. The S_1 segment is the most proximal segment that consists of the convoluted portion of the proximal tubule, and contains epithelial cells with tall brush borders, well-developed lysosome systems, and numerous basally located mitochondria. The S_2 segment extends from the end of the convoluted tubule to the beginning of the straight segment; its epithelial cells have shorter brush borders, fewer mitochondria, and fewer lysosomes than S_1 cells. The S_3 segment consists of the remaining distal straight segment of the proximal tubule and extends into the outer reaches of the medulla. The S_1 and S_2 segments have higher oxygen consumption, sodium/potassium ATPase activity, and gluconeogenic capacity, whereas the S_3 segment has higher transport capabilities for secretion of certain compounds (e.g., organic acids) and is the primary site for metabolic activation of some toxicants (Castro et al., 2008).

The proximal tubule functions in the passive reabsorption of water and active reabsorption of sodium and potassium (via sodium/potassium ATPase pumps), as well as other solutes, including calcium, phosphorus, bicarbonate, glucose, amino acids, proteins, and various xenobiotics (Rouse and Suki, 1985). Each segment of the proximal tubule has a different range of capacity for reabsorption of various solutes; for instance, the S_1 segment reabsorbs a higher percentage of bicarbonate, glucose, amino acids, and low-molecular-weight proteins, whereas the S_2 segment reabsorbs more calcium and phosphorus. Ultimately, the proximal tubule reabsorbs 60%−90% of solute and water that was filtered through the glomerulus. Excretory functions of the proximal tubule include the active

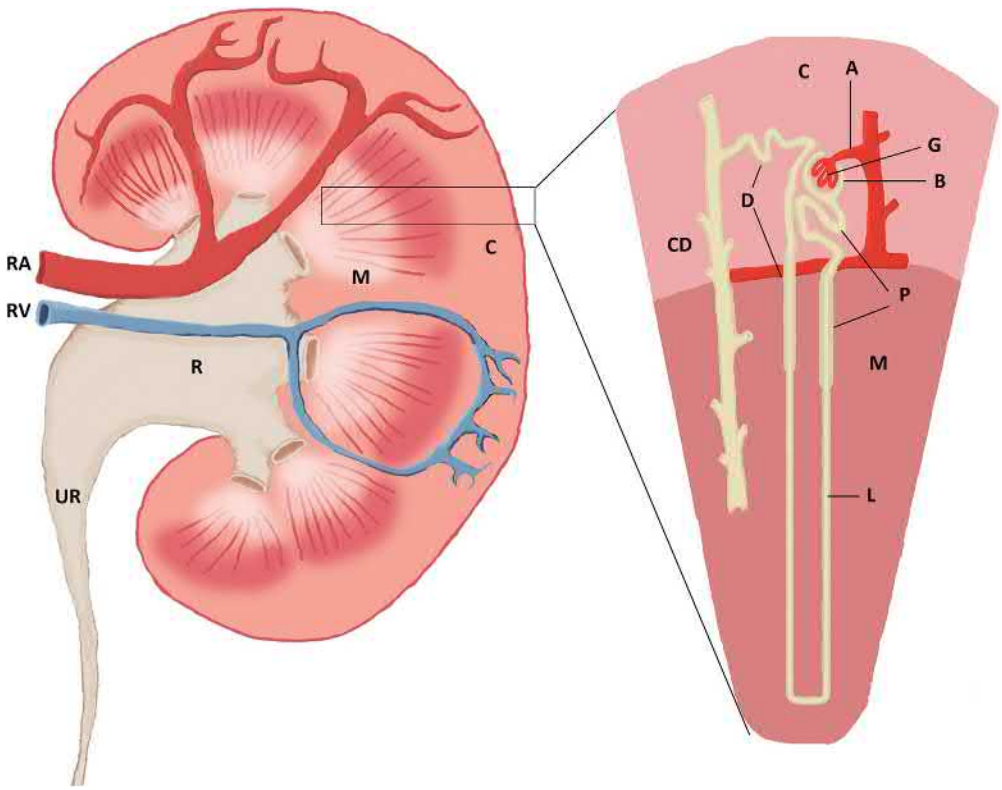

FIGURE 41.1 Diagrammatic view of the kidney showing the relationship of the cortex (C), medulla (M), renal pelvis (R), renal artery (RA), renal vein (RV), and ureter (UR). Enhanced view of microanatomy of the classical nephron. *A*, Afferent arteriole; *B*, Bowman's capsule; *CD*, collecting duct; *D*, distal tubule; *G*, glomerulus; *L*, loop of Henle; *P*, proximal tubule.

secretion of weak organic anions and cations. The absorptive and secretory capacities of the various proximal tubule segments can be induced or inhibited because of pathological or physiological alterations in filtrate composition.

Leaving the proximal tubule, filtrate enters the loop of Henle, which is composed of a thin-walled descending limb and a thick-walled ascending limb that extends to the level of the outer medullary region. Countercurrent exchange mechanisms within the loop result in reabsorption of approximately 20% of filtered water and 25% of filtered sodium and potassium (Schnellmann and Klaassen, 2008). The loop resides largely within the poorly oxygenated renal medulla and contains ATPases with high oxygen demand, making the loop of Henle especially susceptible to hypoxic injury (Brezis et al., 1984). The macula densa sits at the junction of the ascending loop of Henle and the proximal aspect of the distal tubule, in close proximity to the nephron's selfsame afferent arteriole, permitting feedback between macula densa and arteriole. The macula densa "reads" the intratubular solute concentrations and provides feedback to the afferent arteriole, resulting in vasoconstriction or vasodilation that decreases or increases, respectively, the glomerular filtration rate (GFR), thus regulating fluid loss and urine production. The final segment of the distal tubule along with the collecting duct function to reabsorb most of the remaining intraluminal electrolytes and water as needed to regulate the volume and composition of the urine. Collecting ducts progressively intersect and anastomose toward the renal papilla and ultimately empty into the renal calyx, renal pelvis, or ureter, depending on species. Peristaltic action of the ureter propels urine toward the urinary bladder for temporary storage and elimination via the urethra.

In addition to its role in regulation of waste excretion and water/electrolyte balance, the kidney secretes a variety of hormones and regulatory peptides vital for normal systemic homeostasis. Secretion of erythropoietin by renal peritubular interstitial cells promotes red blood cell formation; significant chronic renal disease is often associated with anemia because of decreases in erythropoietin secretion. Renin secreted from the juxtaglomerular cells increases systemic and renal blood pressure and aldosterone release. Prostaglandins and prostacyclin are produced by a variety of renal cells and aid in regulation of renal vascular tone, mesangial contractility, and processing of water and electrolytes by the renal tubules.

41.2.2 Biotransformation

In addition to its other functions, the kidney also plays an important role in xenobiotic metabolism. Renal tubular epithelium contains a variety of biotransformation enzymes including cytochrome P450 monooxygenases, flavin-containing monooxygenases, reductases, hydrolases, UDP-glucuronosyltransferases, sulfotransferases, glutathione-*S*-transferases, cysteine conjugate β-lyase, methylases, and acetylases (Rankin et al., 2005). Although the majority of biotransformation reactions result in inactive metabolites that are then eliminated via the urine, some biotransformation reactions result in the formation of reactive intermediates or metabolites that can cause nephrotoxicity. For example, the blue-green algal toxin cylindrospermopsin requires bioactivation by cytochrome P450 enzymes for it to become genotoxic (Zegura et al., 2011). Biotransforming enzymes are not evenly distributed throughout the kidney, but rather tend to be concentrated in specific sites of the renal tubular epithelium, thereby resulting in a particular pattern of injury depending on which enzyme system was involved in the formation of toxic intermediates.

The cytochrome P450 superfamily is composed of a large number of member enzymes and is divided into four gene families (CYP1, CYP2, CYP3, and CYP4). Individual enzymes (e.g., CYP1E2) are named by family (e.g., CYP1), subfamily (e.g., E), and individual (e.g., 2) designations. This superfamily of enzymes is of major importance in biotransformation of xenobiotics throughout the body and is most prominently expressed in the liver. Cytochrome P405 expression in the kidney is approximately 10% of that of the liver (Cummings et al., 1999). The highest concentrations of P450 in the kidney are in the renal cortex, with smaller amounts in the medulla (Rankin et al., 2005). There is considerable variability in the amount and activity of individual P450 enzymes within the kidney depending on species, age, and gender. Along the nephron, cytochrome P450 expression is highest within the epithelium of the S_2 and S_3 segments of the proximal tubules, so xenobiotics that are bioactivated by P450 to toxic metabolites will preferentially cause damage to those nephron segments first. Flavin-containing monooxygenases catalyze the oxidation of nitrogen-containing, sulfur-containing, phosphorus-containing, selenium-containing, and other nucleophilic heteroatoms-containing compounds in xenobiotics (Cashman and Zhang, 2002). Six isoforms of flavin monooxygenases (FMO1, FMO2, FMO3, FMO4, FMO5, and FMO6) have been identified and the expression of these monooxygenases within tissues varies depending on species, gender, age, and tissue. Glutathione-*S*-transferases catalyze the conjugation of electrophilic sites in xenobiotics with the tripeptide l-γ-glutamyl-l-cysteinylglycine, thus protecting macromolecules from injury from electrophiles (Rankin et al., 2005). Glutathione-*S*-transferase activity is highest in the proximal tubular cells, although distal tubules do express some activity as well.

41.3 Toxic responses of the urinary system

41.3.1 Acute renal failure

Acute renal failure is the sudden decrease in renal function resulting in retention of nitrogenous wastes, and it is a common manifestation of acute nephrotoxic injury (Schnellmann and Klaassen, 2008; Langston, 2010). "Acute kidney injury" (AKI) is the term that has been suggested as most appropriate for use when discussing renal injury, because it encompasses all phases of kidney insult, ranging from minor elevations of serum chemistry renal values to anuric renal failure (Karajala et al., 2010).

The primary manifestation of AKI is decreased GFR resulting in excess accumulation of nitrogenous wastes in the blood (azotemia). Decreased GFR can result from prerenal, renal, or postrenal etiologies. Decreased cardiac output, hypovolemia, and renal vasoconstriction are common prerenal events that can decrease GFR. Postrenal causes of reduced GFR include obstruction of renal tubules or of the lower urinary tract by casts or crystals. Renal tubular injury, glomerular injury, interstitial renal disease (e.g., inflammation, neoplasia), and renal vascular compromise are all primary renal factors that can lead to decreased GFR. In humans, 20%−80% of AKI is attributable to prerenal factors, 10%−45% is attributable to primary renal factors, and postrenal factors cause 5%−15% of AKI (Langston, 2010). Of primary renal factors, ischemia/reperfusion and nephrotoxicosis are thought to be responsible for more than 90% of AKI cases in humans (Schnellmann and Klaassen, 2008).

Damage to kidneys by nephrotoxicants occurs through a variety of different mechanisms, including: direct injury to renal tubular epithelium leading to epithelial cell necrosis with sloughing and obstruction of tubules by cellular debris (tubular casts); detachment of lethally injured cells from the basement membrane resulting in back-leakage of filtrate across the exposed basement membrane and adherence of detached cells to sublethally injured cells still attached to the basement membrane causing lumen obstruction; renal vasoconstriction resulting in hypoxia and ischemic necrosis of renal structures; damage to the glomerular filtration barrier; and impairment of renal healing and repair (Counts et al., 1995). Most nephrotoxic agents cause injury at the level of the renal tubules, and many toxicants will target specific tubule segments.

Clinical effects of AKI generally do not become apparent until GFR is approximately 40% of normal and nitrogenous wastes have accumulated (Khan et al., 2013). Depending on the individual and the dose of toxicant received, this may take several hours to a few days after an acute toxic insult, making nephrotoxicants a poor choice of chemical weapon if the goal is to rapidly disable an opponent. The clinical signs of acute renal injury and/or failure can include polydipsia, nausea or vomiting, lethargy, anorexia, weakness, dehydration, and polyuria/oliguria/anuria. More severe cases may have halitosis, oral ulceration, abdominal (renal) pain, palpably enlarged kidneys, and cardiac arrhythmias. Clinical laboratory abnormalities indicative of AKI include elevations in blood urea nitrogen (BUN) and serum creatinine (azotemia), hyperphosphatemia, hyperkalemia or hypokalemia, and metabolic acidosis. *Uremia* is the term used when azotemia is accompanied by typical clinical signs of AKI. Advanced cases of uremia may present with gastrointestinal ulceration, poorly or nonregenerative anemia, peripheral neuropathy, encephalopathy, metastatic mineralization, and cardiac dysfunction.

The ability of the kidney to heal after an acute toxic insult is dependent on several factors, including the dose and type of toxicant, the amount of functional kidney remaining, the presence and severity of secondary uremic conditions (e.g., soft tissue mineralization), and the degree of medical intervention and supportive care provided during the acute crisis. Mild to moderate renal tubular injury with retention of tubular basement membrane has a reasonable prognosis for tubular regeneration provided that supportive care is administered until tubules have had a chance to recover. In uncomplicated acute tubular injuries, regeneration of epithelial cells generally begins approximately 7−10 days after the renal insult; in mild cases, full recovery of architecture may occur within 2−3 weeks, with longer recovery periods being required for more severe renal injury (Maxie and Newman, 2007). In situations in which nephrons have been fully obstructed by cellular debris or crystals, or if basement membrane integrity is lost, regeneration may be incomplete, resulting in long-term renal insufficiency and/or progression to chronic renal failure (CRF).

41.3.2 Chronic renal failure

The kidneys have a large compensatory capacity to adapt to injury that results in loss of functional renal mass, with potential increases in GFR by 40%−60% in nephrons not directly affected by the insult (Schnellmann and Klaassen, 2008). Biochemical evidence of renal injury generally is not detectable using current renal parameters (i.e., BUN, serum creatinine) until 50%−70% decreases in GFR occur. For these reasons, significant renal injury may occur before any clinically detectable evidence of renal insufficiency develops. CRF is a common result of long-term exposure to toxicants, and many of the alterations found when CRF is finally diagnosed are related to secondary compensatory changes triggered by the initial injury. Toxicant-induced nephron loss causes a decrease

in overall GFR, which triggers an increase in blood flow and pressure to surviving nephrons in an attempt to reestablish normal whole-kidney GFR. Increased glomerular pressure can contribute to degenerative changes such as glomerular sclerosis, tubular atrophy, and interstitial fibrosis, which, in turn, further the progression of renal injury (Brenner et al., 1982). Intraglomerular hypertension results in glomerular hypertrophy, hyaline deposition within glomerular capillary walls, mesangial dysfunction, microaneurysm formation, and thrombosis attributable to endothelial injury (Polzin, 2010). As the glomerulus expands, focal denudation of the GBM occurs, allowing leakage of larger proteins into the glomerular filtrate (proteinuria). Other factors such as cytokines expressed by inflammatory cells, elaboration of reactive oxygen species (ROS), lipid accumulation, increased extracellular matrix deposition, and tubulointerstitial injury contribute to the progression toward CRF (Schnellmann and Klaassen 2008).

Clinical effects associated with CRF include uremia, gastrointestinal disorders (uremic gastritis, uremic enterocolitis), polyuria, polydipsia, nocturia, dehydration, atrial hypertension, peripheral neuropathy, uremic encephalopathy, myopathy, platelet dysfunction, cachexia, and hypokalemia (Polzin, 2010). Edema caused by retention of sodium and water, renal secondary hyperparathyroidism, and anemia caused by decreased erythropoietin synthesis are common manifestations of CRF. Immunosuppression is a potential complication of CRF in humans, and studies of dogs with CRF have shown impaired immunological function as well (Kravola et al., 2010). Patients with CRF may show few outward signs of illness until late in the course of their disease; in some instances, acute decompensation may occur during periods of physical or emotional stress (Hosseininejad and Hosseini, 2008). Lesions of CRF include kidneys that are small and irregular in shape with uneven capsular surfaces. On cut section, pale streaks (fibrosis) may be seen within the interstitium and the parenchyma may be gritty on cutting because of mineralization and/or crystal deposition.

41.3.3 Patterns of toxic injury

Identification of the target site of action of nephrotoxicants can assist in determining the functional impact and potential mechanism of toxicity. Compounds that are directly toxic to cells may cause injury to the glomerulus or the S_1 segment of the proximal tubule as they first enter the nephron. Conversely, direct-acting toxicants may be dilute in the initial ultrafiltrate and may not cause injury until they reach the more distant nephron, where they may reach toxic concentrations as water is gradually reabsorbed and/or the pH changes. Some toxicants require bioactivation to exert their toxic effects and may cause site-specific injury to the segments of the nephron where those bioactivation processes are most active. Fig. 41.2 illustrates the distribution of lesions within the nephron and lower urinary tract that can be induced by chemical warfare agents.

41.3.4 Glomerular injury

The glomerular capillaries are the first component of the nephron to be exposed to bloodborne toxicants. The glomerular cells, matrix, and mesangium are susceptible to toxic injury by several different mechanisms, including direct injury to cellular components, formation of oxygen-derived free radicals, disruption of extracellular substrates (e.g., basement membranes), immune-mediated injury, and disruption of renal hemodynamics (Khan et al., 2013). Direct injury or injury secondary to reactive oxygen intermediates can result in endothelial loss, glomerular podocyte injury, and necrosis of mesangial cells and substrate (mesangiolysis). Further damage could occur secondary to cytokines released by inflammatory cells responding to the site of injury. Alterations in the GBM secondary to cellular injury can result in disruption of the glomerular filtration barrier, leading to proteinuria. Deposition of an immune-mediated reaction to various toxicants (e.g., mercury, gold salts) can result in thickening of the GBM, disrupting the glomerular filtration barrier and leading to membranous glomerulonephropathy.

41.3.5 Proximal tubular injury

Tubular injury is the most common pattern of renal injury induced by toxicants and the proximal tubule is most frequently affected by nephrotoxicants (Schnellmann and Klaassen, 2008). Damage to the proximal tubule may occur because of direct damage from toxicants, metabolic activation of toxicants, ischemia–reperfusion, or physical or chemical disruption of endothelium and/or basement membrane. The S_1 segment is the most vulnerable to injury from toxicants that exert direct injury because the epithelium in this area is exposed to the toxicant first. The proximal convoluted tubule epithelium is actively involved in endocytosis of various compounds that bind to the brush border, sequestering the compounds in phagolysosomes. When this process is overwhelmed by the presence of certain toxicants (e.g., uranyl ion), loss of phagolysosome membrane integrity occurs, resulting in lysosomal leakage and cell injury or necrosis (Khan et al., 2013). This type of injury is most commonly associated with the S_1 and S_2 segments of the proximal tubule. In contrast, the straight segment (S_3) is most susceptible to injury by metabolic activation, transporter-associated accumulation, and ischemia–reperfusion.

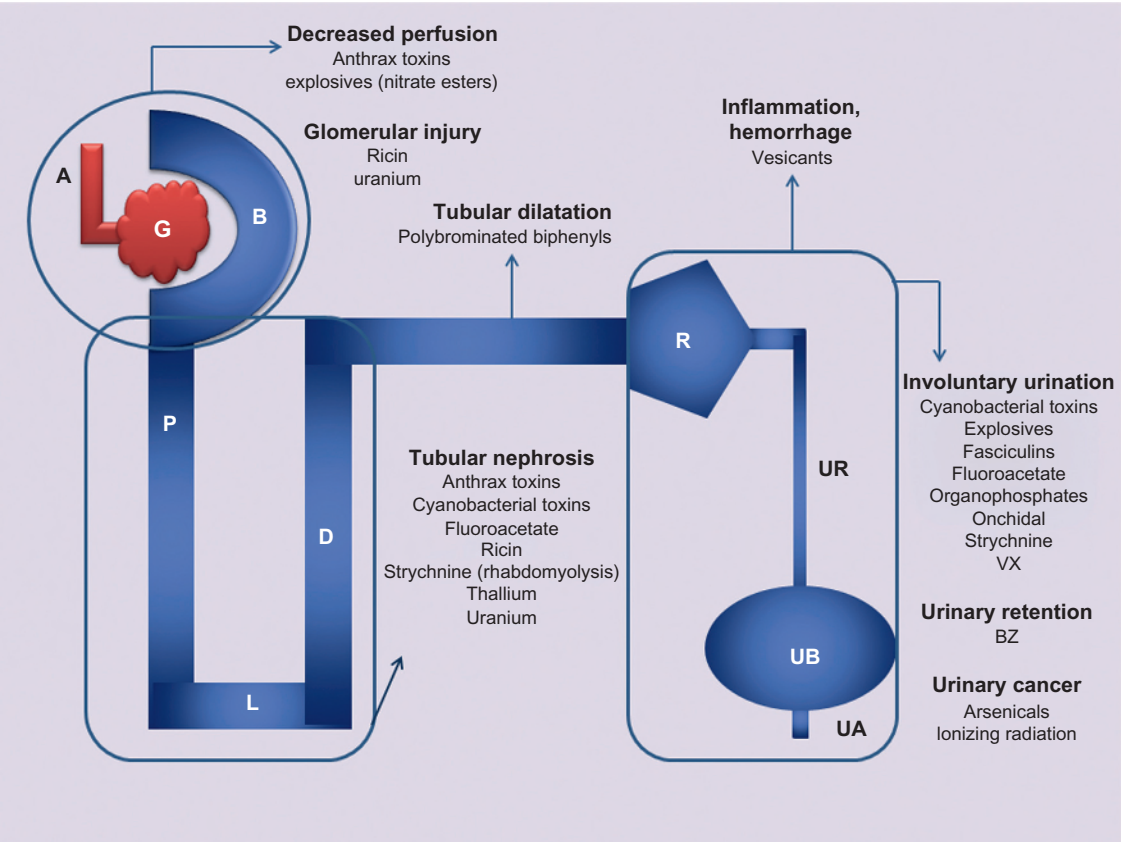

FIGURE 41.2 Cartoon of the nephron and lower urinary tract showing sites of injury caused by various chemical and biological warfare agents. *A*, afferent arteriole; *B*, Bowman's capsule; *C*, collecting ducts; *D*, distal tubule; *G*, glomerulus; *L*, loop of Henle; *P*, proximal tubule; *R*, renal pelvis; *UA*, urethra; *UB*, urinary bladder; *UR*, ureter.

Various metabolic derangements, such as severe acid–base imbalances, induced by toxicants can result in injury to renal tubules. Prolonged muscle activity from tremors or convulsions (e.g., from nerve agents) may result in rhabdomyolysis with myoglobin pigment causing renal tubular damage (Hamilton et al., 1989). Similarly, intravascular hemolysis that occurs during a chemical warfare toxidrome can result in hemoglobinuric nephropathy with resulting renal tubular injury. Decreased cardiac output, hypovolemia, and anemia can result in ischemia of renal tissue and precipitate tubular injury; renal ischemia can also occur secondarily to toxicosis from agents that alter oxygen delivery to tissues (e.g., cyanide).

In humans and dogs, the renal tubules and interstitium are related in such a way that significant injury to one results in a reaction in the other; for this reason, the terms "tubulointerstitial disease" and "chronic interstitial nephritis" are often applied to lesions found in the kidneys of these species (Khan et al., 2013). The latter term acknowledges the fact that, in advanced tubulointerstitial disease, it is difficult to determine which injury came first, that to tubules or to interstitium.

41.3.6 Distal nephron/renal papillary injury

Toxic injury to the distal nephron is relatively uncommon, and injury to this area generally manifests as decreased urine-concentrating ability or as defects in acid secretion resulting in metabolic acidosis (Khan et al., 2013). Injury to the renal papilla is most commonly seen with toxicants that impede blood flow to this normally poorly perfused area. Direct injury to renal papillae from renally excreted irritants (e.g., vesicants such as cantharidin) may also occur.

41.3.7 Lower urinary tract

Toxicants that cause injury to the ureters, urinary bladder, and urethra tend to be those that directly interact with the urothelium (Cohen, 2013). Direct cytotoxicity to urothelium by vesicants such as cantharidin can result in necrosis, ulceration, and hemorrhage. Ionizing radiation and genotoxic chemicals can damage DNA within the regenerative layer of the urothelium, inducing mutations and increasing the potential for development of urinary tumors. Not all toxic effects on the lower urinary tract are

related to cellular injury. Psychotropic or neurotoxic agents can affect neuromuscular control of the bladder, resulting in the involuntary retention or voiding of urine.

41.4 Toxic effects of chemical warfare agents

Very few compounds selected as agents of chemical warfare have significant and direct effects on the urinary system, and any injury to the kidney can be considered an unintended consequence of most chemical weapons. Indirect renal injury can occur because of a variety of effects of chemical weapons, including hemodynamic abnormalities causing decreased renal perfusion (e.g., shock, hypotension), acid–base derangements, interference with normal renal cellular function, or induction of pigmenturia attributable to rhabdomyolysis or hemolysis.

41.4.1 Vesicants

Vesicants are agents that produce chemical burns on body surfaces. Phosgene oxime, an urticariant, is not a true vesicant because it does not produce blisters but is included in this section. Through their action on the skin, respiratory tract, and eyes, these agents tend to incapacitate rather than kill, although systemic involvement and death can occur with severe exposures or because of secondary complications such as bacterial infections, shock, or multiorgan failure.

The biochemical mechanisms of injury from vesicant agents are poorly understood. Mustards [bis-(2-chloroethyl) sulfide] are thought to act as alkylating agents that form highly reactive electrophiles that combine with nucleophilic sites on cellular macromolecules to form stable adducts that interfere with normal function of cells and disrupt the epidermal–dermal junction (Hurst et al., 2008). Once tissue injury has been established, activation of massive local inflammatory response leads to vesicle development. Depletion of glutathione may also contribute to the vesicant injury through loss of protection against oxygen-derived free radicals and lipid peroxidation, resulting in a "snowballing" effect of cellular damage. The biochemical mechanism of action of the arsenical lewisite (*b*-chlorovinyldichloroarsine) may involve interactions with enzymatic sulfhydryl groups, resulting in inhibition of the pyruvate dehydrogenase complex, leading to inactivation of carbohydrate metabolism. As with mustards, depletion of glutathione resulting in increased lipid peroxidation and ROS generation may also play a role in lewisite-mediated cellular injury. Phosgene oxime may act by direct necrotizing effects of chlorine, by enzymatic inactivation of target cells, and/or by activation of macrophages, recruitment of neutrophils, and production of ROS, although the exact mechanism remains obscure (Hurst et al., 2008).

Renal lesions with vesicant exposures are not commonly encountered and, in many cases, may be nonspecific secondary effects attributable to multiorgan failure associated with fatal exposures. Mustards have been reported to cause hemorrhagic nephritis, oliguria, anuria, albuminuria, and casts in mortally injured victims (Papirmeister et al., 1991). Microscopic lesions associated with sulfur mustard victims included calcified and hemoglobin-containing intratubular casts (Alexander, 1947). Although lewisite is an arsenical vesicant, renal lesions typical for inorganic arsenic toxicosis have not been reported in laboratory species or human patients exposed to lewisite (Hurst et al., 2008). No specific renal lesions have been reported with exposure to phosgene oxime. For further details on the toxicology of vesicants/blister agents, readers are referred to Chapter 11, Blister agents.

41.4.2 Nerve agents

Nerve agents classically include the organophosphorus (OP) compounds (e.g., soman, sarin, cyclosarin, tabun, and VX) originally developed as insecticides, but repurposed and refined for use as warfare agents as their extreme toxicity was recognized (Sidell et al., 2008). Although developed and stockpiled for use in Germany during World War II, nerve agents were not used on the battlefield until 50 years later during the Iran–Iraq War. Since then, nerve agents have been used both in battle and in terrorist attacks, such as the 1995 release of sarin on commuter trains in Japan that killed 12 people. Onchidal, an anticholinesterase mollusk toxin, has similar clinical effects to the OP nerve agents and is considered here under the term "nerve agent" (Abramson et al., 1989).

Nerve agents act by binding and inhibiting acetylcholinesterase (AChE), an enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh). The cholinergic system is the only known system to terminate the action of a neurotransmitter via enzymatic cleavage. When anticholinesterase agents such as OPs and onchidal disable AChE, ACh accumulates within the synapse, resulting in continued stimulation of cholinergic pathways and development of the classic nicotinic and muscarinic signs (Anadon et al., 2015). Urinary system signs associated with anticholinesterase poisoning include involuntary urination because of cholinergic stimulation of bladder musculature. Acute renal injury has occasionally been reported after anticholinesterase poisonings and may be attributed to hemodynamic dysfunction leading to shock and hypovolemia during the cholinergic crisis, which, in turn, results in decreased renal perfusion (Bloch-Shilderman

and Levy, 2007). Because the mechanism of anticholinesterase poisoning is biochemical, few characteristic gross lesions occur beyond nonspecific organ congestion and the presence of increased bronchial secretions (Meerdink, 2004). Histopathologic evidence of skeletal muscle necrosis, myocardial hemorrhage and necrosis, and neuronal necrosis within the hippocampus, cerebral cortex, amygdala, and thalamus have been reported after severe OP intoxication (Gupta et al., 1987a,b, 1991, 2013).

41.4.3 Depleted uranium

Depleted uranium (DU) is previously enriched uranium that has had its radioactivity largely spent and, as such, is a very weak alpha and gamma emitter (Gwaltney-Brant, 2013). Lesions produced by DU are thereby related to the metal itself rather than any emitted radiation. Uranium is poorly absorbed orally, and absorption via inhalation requires uranium to be soluble and of small size ($<10\ \mu m$). Once absorbed, uranium is converted to uranyl ion that complexes with citrate, bicarbonate, or plasma proteins and is distributed via the blood, ultimately accumulating in bone and in the proximal tubules of the kidney. In the proximal tubular epithelium, uranyl ion is cleaved from its complex and causes damage to renal tubules and glomeruli, resulting in proteinuria, glucosuria, aminoaciduria, and, with higher exposure levels, acute renal failure. Glomerular lesions include endothelial swelling and necrosis, glomerular sclerosis, and disruption of glomerular fenestrae. Tubular epithelium becomes vacuolated and necrotic, and over time a mosaic of degenerating and regenerating tubules and glomeruli can be seen along with interstitial inflammation and fibrosis. On removal of uranium exposure, tubules and glomeruli may fully regenerate, provided that fibrosis has not developed.

41.4.4 Thallium

Thallium is a heavy metal that has historically been used as a pesticide and as a therapeutic agent; it has also been used illegally as an agent for suicide attempts and intentional poisonings of small groups of people (Thompson, 2015). Thallium is rapidly absorbed via most routes and is widely distributed throughout the body, although the highest levels occur in the skin. Thallium substitutes for potassium in many cellular enzymatic and transport systems, causing widespread disruption of cellular function. Clinically, the classic syndrome of thallium poisoning involves gastroenteritis, polyneuropathy, and alopecia. Renal injury may occur secondarily and is characterized by elevations in BUN and urine protein levels. Histopathological lesions in the kidney include necrosis of epithelium of the loop of Henle, degeneration and necrosis of proximal convoluted tubules, and stromal edema. On electron microscopy, degenerative changes were seen in mitochondria, microvilli, and endoplasmic reticulum (Danilewicz et al., 1979). For further details on thallium toxicosis, see Chapter 20, Thallium.

41.4.5 Ricin

Ricin is a lectin isolated from the seeds of the castor bean plant (*Ricinus communis*) that acts as a ribosome-inactivating protein, enzymatically depurinating adenine residues of 28S ribosomal RNA and irreversibly arresting protein synthesis (Lapadula et al., 2013). The toxicity of ricin has been known for centuries, and more than 750 human cases of intoxication have been reported (Poli et al., 2007). Its potency, worldwide availability, and ease of production have made ricin a biological warfare agent of interest in many countries, but its widespread use as such has not been reported. Ricin has been used as an agent of political assassination, with the most famous incident being the murder of Georgi Markov, a Bulgarian dissident who died a few days after a ricin-impregnated bead was injected into his thigh via a specially modified umbrella (Papaloucas et al., 2008). The fact that the ricin had to be injected into Markov underscores one of the drawbacks in its use as an agent of warfare or terrorism: the toxicity of ricin is largely route-dependent (Poli et al., 2007). Dermal absorption of ricin does not occur to any significant degree; ingestion results primarily in gastrointestinal injury, which is largely survivable if patients receive appropriate therapy. Parenteral administration via injection is not feasible for large-scale use, and efficient inhalational administration requires refinement in manufacturing and delivery methods that make it less desirable as an efficient warfare or terrorism weapon (Schep et al., 2009).

Clinical signs and pathologic lesions in ricin toxicosis are largely route-specific, with ingestion causing primarily gastrointestinal signs (vomiting, diarrhea, abdominal pain) and with inhalation resulting in respiratory signs such as wheezing, pneumonia, and pulmonary edema (Poli et al., 2007). At larger oral or inhaled dosages or with parenteral administration (i.e., injection), more serious systemic effects can develop, including hemorrhagic diarrhea, fever, vascular collapse, hypotension, dehydration, cyanosis, hypovolemic shock, and death after 3 or more days. Liver failure and kidney failure have been reported in human cases with survival beyond several days. Systemic lesions after ricin ingestion by humans include gastrointestinal ulceration and hemorrhage, diffuse nephritis, and necrosis of the liver, spleen, and lymph nodes. Renal lesions associated with ricin toxicosis resemble lesions of hemolytic-uremic syndrome, which is most commonly associated with verocytotoxin-producing *Escherichia coli* (Taylor et al., 1999; Korcheva et al., 2005). In rodents exposed intratracheally to lethal levels of ricin, initial renal injury occurs in the glomerulus, which shows leukocytosis,

accumulation of proinflammatory RNA transcripts, substantial damage to 28S rRNA, and accumulation of fibrin and fibrinogen, resulting in glomerular thrombotic microangiopathy (Wong et al., 2007). Renal tubular degeneration and necrosis develop subsequent to the glomerular injury. Mice exposed to sublethal levels of ricin did not have development of histopathological lesions and 28S rRNA damage within the kidney. In dogs with naturally occurring fatal ricin toxicosis, membranous glomerulonephritis devoid of fibrin deposits and renal tubular degeneration and necrosis have been reported (Roels et al., 2010). For details on ricin toxicosis, see Chapter 28, Ricin.

41.4.6 Anthrax toxins

Anthrax is a zoonotic disease caused by *Bacillus anthracis* that has a long association in human history. Inhalational anthrax, also known as woolsorter's disease because of its association with English woolsorters, is of concern because of its ability to be spread via the aerosol route and its high mortality in humans lacking rapid access to appropriate treatment. A 2001 bioterrorist attack in the United States resulted in the deaths of 5 of 11 individuals who had development of inhalational anthrax. In addition to inhalational anthrax, other forms of anthrax include gastrointestinal anthrax, cutaneous anthrax, and meningitis, with the latter occurring as a complication attributable to bacteremia from any of the other forms of the disease (Purcell et al., 2007).

B. anthracis produces two major toxins, lethal toxin (LT) and edema toxin (ET), which play essential roles in its virulence (Sweeney et al., 2010). As with many bacterial toxins, anthrax toxins are binary in structure, possessing a cell-binding, pore-forming subunit that gains entry into cells, and an enzymatic component that produces the toxic effects. LT is a zinc endopeptidase that cleaves mitogen-activated protein kinases, whereas ET is a potent calmodulin-dependent adenyl cyclase. Although ET is less potent than LT on a molar basis, it produces death more rapidly and is primarily responsible for the renal effects of anthrax (Fioved et al., 2005). ET causes decreased renal perfusion as well as possible direct cytotoxic effects on renal tubular epithelium, leading to increases in BUN and serum creatinine. Histopathologic renal lesions of anthrax include degeneration and necrosis of cortical tubular epithelial cells.

41.4.7 Cyanobacterial toxins

Cyanobacteria produce a variety of toxins, including anatoxins, microcystins, nodularins, and cylindrospermopsin (Patocka et al., 2011; Puschner, 2018). Anatoxin-a and anatoxin-a(s) are not directly nephrotoxic, but their effects on the nervous system (nicotinic and anticholinesterase, respectively) can result in loss of voluntary bladder control and involuntary urination. Microcystins and nodularins are primarily hepatotoxins that cause apoptosis and necrosis of hepatocytes through interference with cytoskeletal structures after acute exposure. Death from acute microcystin toxicosis is attributable to liver failure, with any renal injury generally attributed to terminal multiorgan failure. Chronic administration of microcystins to rats resulted in nephrotoxicity caused by disruption of cytoskeletal structures of the renal tubular cells (Milutinovic et al., 2003). Nodularins have been reported to produce renal lesions after acute exposure (Simola et al., 2012). The lesions were described as radiating streaks of acute tubular necrosis primarily affecting proximal tubular epithelium, with multifocal extensions of necrosis into more distal tubules and collecting ducts. In both microcystin and nodularin toxicosis, decreased renal perfusion may play a role in the renal lesions that develop. Cylindrospermopsin binds to DNA, causing DNA damage, inhibiting protein synthesis, and inducing oxidative damage to cells (Solter and Beasley, 2013). As with microcystins and nodularins, the liver is the primary target of cylindrospermopsin, with renal injury thought to be a combination of direct toxic injury as well as ischemic injury attributable to cardiovascular compromise in terminal stages of toxicosis. Renal lesions include acute degeneration and necrosis of proximal and distal convoluted tubules. In addition to cytotoxicity, cylindrospermopsin can be bioactivated to a genotoxic compound by cytochrome P450 enzymes (Zegura et al., 2011). The genotoxic nature of cylindrospermopsin also makes it a concern as a potential human and animal carcinogen.

41.4.8 Other agents

Many potential agents of chemical warfare have little to no direct injurious effect on the kidney or urinary system, but instead may cause indirect renal injury and/or urinary dysfunction. Nitrate esters used as explosives can cause profound hypotension with the potential to result in renal ischemia secondary to decreased renal perfusion (Gahagan and Wismer, 2012). Metabolic acidosis, proteinuria, glucosuria, and myoglobinuria were reported in a survey of five human cases of cyclonite (C-4) plastic explosive ingestion, and chronic renal insufficiency developed in a dog that recovered from seizures caused by ingestion of cyclonite (Kuccukardali et al., 2003; Fishkin et al., 2008). Chronic or repeated exposure to the riot control agent chloropicrin (PS, nitrochloroform) has been reported to cause kidney injury, but the mechanism or nature of renal injury has not been fully described (Salem et al., 2008). Cattle fed 25 g of polybrominated biphenyls per day for 33–60 days had development of degeneration and necrosis of cells of the collecting ducts and convoluted tubules, whereas those fed

250 mg per day had no development of signs or lesions of toxicosis (Moorhead et al., 1978). Toxic inhalants such as phosgene, chlorine, and hydrogen cyanide do not appear to cause direct injury to the kidney, nor do they appear to cause chronic or long-term renal effects (Tuorinsky and Sciuto, 2008).

Agents that cause neuromuscular dysfunction resulting in fasciculations, tremors, or seizures include compounds such as nerve agents, strychnine, fasciculins, and fluoroacetate. Although none of these have clinically significant direct toxic effect on the kidney, all have the potential to induce involuntary urination during the period of intoxication, and myoglobinuria or hemoglobinuria secondary to severe muscle injury from prolonged tremor or convulsion can cause acute renal tubular degeneration and necrosis. Incapacitants and psychotropic agents such as LSD also can result in loss of voluntary bladder control without causing overt lesions within the urinary system (Ketchum and Salem, 2008). Similarly, agents that produce neuromuscular blockade, paralysis, or unconsciousness, such as tetrodotoxin or botulinum toxin, can cause loss of voluntary bladder control, but cause no direct action on the kidney (Williams et al., 2007; Dembek et al., 2007). Conversely, the antimuscarinic nerve agent 3-quinuclidinyl benzilate (BZ) can cause urinary retention necessitating urethral catheterization to empty the bladder (Barreuto et al., 2006).

41.5 Concluding remarks and future directions

Despite the fact that some nephrotoxic agents (e.g., ethylene glycol) have been used in cases of malicious poisoning of individuals or in cases of suicide, in general, the urinary system makes a poor target for chemical warfare agents whose goal is to rapidly and consistently disable, incapacitate, or kill people on a large scale. Although renal dysfunction can lead to debilitating illness and death of the individual, barriers to the use of nephrotoxins as chemical weapons include delay between exposure and development of clinical signs, difficulty in ensuring delivery of appropriate doses to the targets, and individual variation in response to nephrotoxic agents. Given these barriers, it is not surprising that agents that have been developed to be used for chemical warfare tend to target other organ systems that can accomplish the goal of rapid incapacitation of the opponent more efficiently than primary nephrotoxicants.

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Chapter 42

Impact of chemical warfare agents on the immune system

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42.1 Introduction

Chemical warfare agents (CWAs) are probably the most brutal agents created by mankind for military operations to kill during war as compared to biological and nuclear warfare. Chemical weapons are inexpensive, relatively easy to manufacture, and can incapacitate people by inducing severe pathophysiological changes in various body systems even with small quantities. A United Nations report from 1969 defines CWAs as "chemical substances, whether gaseous, liquid or solid, which might be employed because of their direct toxic effects on man, animals and plants." However, the Chemical Weapons Convention defines chemical weapons as including not only toxic chemicals but also ammunition and equipment for their dispersal. Toxic chemicals are stated to be "any chemical which, through its chemical effect on living processes, may cause death, temporary loss of performance, or permanent injury to people and animals." Normally, they are highly toxic synthetic chemicals that can be dispersed as a liquid (including aerosols), gas, solid, or adsorbed on to particles to become a powder.

A chemical incident is traditionally defined as an unexpected or uncontrolled release of a chemical from its containment. Chemical incidents may be small or large in scale, and can give rise to multiple primary or secondary chemical casualties and fatalities. Chemical agents have been used in war since time immemorial. The use of CWAs in battlefields reached a peak during World War I and the French were the first to use ethylbromoacetate. This was followed by *o*-dianisidine chlorosulfonate, chloroacetate, chlorine, phosgene, hydrogen cyanide, diphenylchloroarsine, ethyl- and methyldichloroarsine, and sulfur mustard, resulting in nearly 90,000 deaths and over 1.3 million casualties (Eckert, 1991). CWAs were most brutally used by the Germans in the gas chambers for mass genocide of Jews during World War II, and have been used intermittently both in war, as in the Iraq−Iran War, as well as in the terrorist attacks in Japanese subway stations. It is estimated that nearly 100,000 US troops may have been exposed to CWAs during operation Desert Storm (Chauhan et al., 2008).

CWAs have been widely condemned since they were first used on a massive scale during World War I. However, they are still stockpiled and used in many countries as they are cheap and relatively easy to produce, and can cause mass casualties. Although the blood agent CK is extremely volatile and undergoes rapid hydrolysis, the degradation of three types of vesicant CWAs, the sulfur mustards, nitrogen mustards, and lewisite, results in persistent products. For example, sulfonium ion aggregates formed during hydrolysis may be persistent and may retain vesicant properties. The nerve agents include the V agent VX as well as three G agents (tabun, sarin, and soman). VX gives rise to two hydrolysis products of possible concern: EA 4196, which is persistent, and EA 2192, which is highly toxic and is possibly persistent under certain limited conditions (Small, 1984). Thus, their long-term persistence in the body may lead to alterations in the immune system of the exposed population. This chapter describes the immunotoxicity of CWAs and gives an insight into the probable mechanisms of such effects.

42.2 The immune system

Immunotoxicity deals with immune dysfunction resulting from exposure of an organism to a xenobiotic and explores the mechanisms underlying these effects in a biological system. The immune dysfunction may take the

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form of immunosuppression or alternatively, exaggerated immune reaction, namely allergy, autoimmunity, or any number of inflammatory-based diseases or pathologies. Immunotoxicity adversely affects the functioning of both local and systemic immune systems, which gets activated on exposure to toxic substances including CWAs. Observations in humans and animal studies have clearly demonstrated that a number of environmental and industrial chemicals can adversely affect the immune system. Immunosuppression may lead to the increased incidence or severity of infectious diseases or cancer, since the immune system's ability to respond adequately to invading agents is suppressed. Toxic agent-induced immunostimulation can cause autoimmune diseases, in which healthy tissue is attacked by an immune system that fails to differentiate self-antigens from foreign antigens. For example, the pesticide dieldrin induces an autoimmune response against red blood cells, resulting in hemolytic anemia.

The immune system is evolved in multicellular organisms to protect them from various pathogens. The immune system is composed of several organs, cells, and noncellular components which act in an interrelated manner to protect the host against foreign organisms and chemical substances. A clear understanding of each of the many players involved will help one appreciate the intricate coordination of the immune system. The immune system participates in the mechanisms responsible for the maintenance of homeostasis and an altered immune system reflects the adverse changes in both internal and external microenvironments. The immune system protects organisms against pathogens or other innocuous substances like pollens, chemicals, indoor molds, potential food allergens, and environmental agents, and acts as layered defenses of increasing specificity. Most simply, physical barriers (e.g., skin) prevent pathogens and xenobiotics from entering the organism. If they breach these barriers, the innate immune system provides an immediate but nonspecific response. However, if pathogens successfully evade the innate response, there is a third layer of protection, that is, the adaptive immune system, which is activated by the innate response. Here, the immune system adapts during an infection to improve its recognition of the pathogen and its response is then retained after the pathogen or xenobiotic has been eliminated. This immunological memory allows the adaptive immune system to respond faster, with a stronger attack each time the same insult is encountered (Kindt et al., 2007).

The immune system protects organisms from infection with layered defenses of increasing specificity. The layered defense includes mechanical, chemical, and biological barriers which protect organisms from toxic substances. Skin, a mechanical barrier, acts as the first line of defense against infection. In the lungs, coughing and sneezing mechanically eject pathogens and other irritants from the respiratory tract, while mucus secreted by the respiratory and gastrointestinal tract traps and entangles microorganisms and other toxins (Boyton and Openshaw, 2002). Chemical barriers also protect against infection. The skin and respiratory tract secrete antimicrobial peptides such as the β-defensins. Enzymes such as lysozyme and phospholipase A2 in saliva, tears, and breast milk are also antibacterials (Moreau et al., 2001). In the stomach, gastric acid and proteases serve as powerful chemical defenses against ingested pathogens.

There are two interconnected systems of defense mechanisms: innate and adaptive immunity. These two systems collaborate to protect the body against foreign invaders.

42.2.1 The innate immune system

Innate immunity includes built-in molecular and cellular mechanisms that are encoded in the germline and are evolutionarily more primitive, aimed at preventing infection or quickly eliminating common invaders, in a nonspecific manner. This means that the cells of the innate system recognize, and respond in a generic way, but do not confer long-lasting or protective immunity to the host. The innate immune response was initially dismissed by immunologists as it was thought to provide a temporary holding of the situation until a more effective and specific adaptive immune response develops. However, it has now become clear that it plays an important role as a dominant system of host defense in most organisms (Litman et al., 2005). The major function of the innate immune system is to recruit immune cells to sites of infection and inflammation. Inflammation is one of the first responses of the immune system to infection or irritation through the production of cytokines. These cytokines released by injured cells serve to establish a physical barrier against the spread of infection. Several chemical factors are produced during inflammation, for example, histamine, bradykinin, serotonin, leukotrienes, and prostaglandins, which sensitize pain receptors, cause vasodilation of the blood vessels, and attract phagocytes.

The inflammatory response during innate immunity is characterized by the redness, heat, swelling, pain, and possible dysfunction of the organs or tissues involved. The fluid exudate contains the mediators for four proteolytic enzyme cascades: the complement system, the coagulation system, the fibrinolytic system, and the kinin system. The exudate is carried by lymphatics to lymphoid tissue, where the product of foreign organisms can initiate an immune response. The activation of the complement cascade helps to identify the invading substance, activate

cells, and promote clearance of dead cells by specialized white blood cells (WBCs). The cascade is composed of nine major components, designated C1−C9, which are plasma proteins synthesized in the liver, primarily by hepatocytes. These proteins work together to trigger the recruitment of inflammatory cells. One of the main events is the splitting of the C3, which gives rise to various peptides. One of them, C3a (anaphylatoxin), can stimulate mast cells to secrete chemical mediators and another, C3b (opsonin), can attach to the surface of a foreign body and facilitates its ingestion by WBCs. C5 is a powerful chemotactic of white cells and causes release of mediators from mast cells. Later components from C5 to C9 assemble in a sequence at the surface of bacteria/xenobiotics and lead to their lyses, ridding the body of neutralized antigen−antibody complexes. The main events of this system can also be directly initiated by the principal enzymes of the coagulation and fibrinolytic cascade, thrombin and plasmin, and by enzymes released from WBCs. Further, an innate immune system leads to the activation of an adaptive immune system.

42.2.2 The adaptive immune system

A second form of immunity, known as adaptive immunity, is much more attuned to subtle molecular differences. The adaptive immune system is composed of highly specialized, systemic cells and processes that eliminate pathogenic challenges and provide the ability to recognize and mount stronger attacks each time the same pathogen is encountered. Antigen specificity requires the recognition of specific "nonself" antigens during a process called antigen presentation. The ability to mount these immune responses is maintained in the body by "memory cells." The response is slow to develop, but is much more antigen specific. The cells of the adaptive immune system are special types of leukocytes, B cells and T cells, which constitute about 20%−40% of WBCs. The peripheral blood contains 20%−50% of circulating lymphocytes and the rest move within the lymphatic system (Kindt et al., 2007). B cells and T cells are derived from the same pluripotential hematopoietic stem cells (HSCs) in the bone marrow, and are indistinguishable from one another until after they are activated. B cells play a large role in the *humoral immune response*, whereas T cells are intimately involved in *cell-mediated immune responses*. B cells derive their name from the bursa of Fabricius, an organ unique to birds, where the cells were first found to develop. However, in nearly all other vertebrates, B cells (and T cells) are produced by stem cells in the bone marrow (Kindt et al., 2007). T cells are named after thymus where they develop and through which they pass. In humans, approximately 1%−2% of the lymphocyte pool recirculates each hour to optimize the opportunities for antigen-specific lymphocytes to find their specific antigen within the secondary lymphoid tissues. Both B cells and T cells carry receptor molecules that recognize specific targets.

Naive T cells continually recirculate between the blood and lymph systems. During recirculation, naive T cells reside in secondary lymphoid tissues such as lymph nodes. If a naive cell does not encounter antigen in a lymph node, it exits through the efferent lymphatics, ultimately draining into the thoracic duct and rejoining the blood. It is estimated that each naive T cell recirculates from the blood to the lymph nodes and back again every 12−24 h. Because only about 1 in 10^5 naive T cells is specific for any given antigen, this large-scale recirculation increases the chances that a naive T cell will encounter the appropriate antigen. T cells express a unique antigen-binding molecule, the T-cell receptor (TCR), on their membrane. There are two well-defined subpopulations of T cell: T helper (T_H) and T cytotoxic (T_C) cells. They can be distinguished from one another by the presence of either CD4 or CD8 membrane glycoproteins on their surfaces. T cells displaying CD4 generally function as T_H cells whereas those displaying CD8 function as T_C cells. T cells recognize a "nonself" target, such as a pathogen, only after antigens have been processed and presented in combination with a "self" receptor called a major histocompatibility complex (MHC) molecule. T_C cells only recognize antigens coupled to class I MHC molecules, while T_H cells only recognize antigens coupled to class II MHC molecules. Naive T_H cells are activated only by dendritic cells, whereas memory T_H cells can be activated by macrophages, dendritic cells, and B cells (Thokchom et al., 2017).

B cells are the major cells involved in the creation of antibodies that circulate in blood plasma and lymph, known as humoral immunity. The function of a B cell is to secrete antibodies capable of binding to any organism or molecule that poses a threat to the host. Like the T-cell receptor, B cells express a unique B-cell receptor (BCR), in this case an immobilized antibody molecule. The BCR recognizes and binds to only one particular antigen. A critical difference between B cells and T cells is how each cell "sees" an antigen. T cells recognize their cognate antigen in a processed form—as a peptide in the context of an MHC molecule—while B cells recognize antigens in their native form. Once a B cell encounters its cognate (or specific) antigen and receives additional signals from a helper T cell, it further differentiates into an effector cell, known as a plasma cell.

A single plasma cell is capable of secreting antibody molecules actively from a few hundred to more than a thousand molecules of antibody per second and they are

responsible for humoral immunity. Antibodies (or immunoglobulin, Ig) are large Y-shaped proteins used by the immune system to identify and neutralize foreign objects. In mammals there are five types of antibody: IgA, IgD, IgE, IgG, and IgM. Differing in biological properties, each has evolved to handle different kinds of antigens. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement cascade. About 10% of plasma cells will survive to become long-lived antigen-specific memory B cells (Lu and Kacew, 2002). The memory B cells circulates through the lymphoid organs and remain poised for subsequent stimulation and respond quickly if the same foreign body reinfects the host and prepares the immune system for future challenges.

Although immunotoxicology is a relatively new field, a considerable amount of data has accumulated during the past few years on immunotoxicity of certain xenobiotics. The majority of the research thus far carried out has been on environmental contaminants. Thus, from the defense point of view considerable work is required to investigate the immunotoxicity of several chemicals and some bacterial and fungal toxins which may be potential CWAs. Furthermore, there are several chemicals used in the defense industry to which defense industrial workers may be constantly exposed. These chemicals, following low-level exposure to humans and animals, may cause immunological alterations. Thus studies on such chemicals are being conducted to understand the potential risks of such exposure on the host's defense as well as the cellular and molecular mechanism of such immunomodulatory action. A number of animal models have been developed and validated to detect the chemical-induced direct immunotoxicity.

42.3 Targets of immunotoxicity

42.3.1 Effects on precursor stem cells

All functionally specialized, mature blood cells (red blood cells, granulocytes, macrophages, dendritic cells, and lymphocytes) arise from a single cell type, the HSC. The process by which HSCs differentiate into mature blood cells is called hematopoiesis. Two primary lymphoid organs are responsible for the development of stem cells into mature immune cells: the bone marrow, where HSCs reside and give rise to all cell types; and the thymus, where T cells complete their maturation. All leukocyte lineages originate from these stem cells, but once distinct subsets of leukocytes are established, their dependence on replenishment from the bone marrow differs vastly. The turnover of neutrophils is very rapid, that is, more than 10^8 neutrophils enter and leave the circulation in a normal adult daily so there is dependence on new formation in the bone marrow. In contrast, macrophages are long-lived and have little dependence on new formation of precursor cells. The adaptive immune system, comprising antigen-specific T and B lymphocytes, is almost completely established around puberty and is therefore essentially bone marrow independent in the adult.

As a consequence of their high proliferation rate, stem cells in the bone marrow are likely to be extremely vulnerable to cytostatic drugs and chemicals like CWAs. Lineages like neutrophils with rapid turnover will be most vulnerable and will be affected first by such treatments/exposures. After prolonged exposure, macrophages and T or B cells of the adaptive immune system are also suppressed. Recognizing immunomodulation mechanisms produced by CWAs is a platform for identifying the best therapeutic and management approaches to check deleterious effects on the biological system.

42.3.2 Effects on maturation of lymphocytes

The thymus is a primary lymphoid organ where T cells mature. T-cell development is not complete until the cells undergo selection in the thymus. T lymphocytes mature in the thymus by a very complex selection process that takes place under the influence of the thymic microenvironment and ultimately generates an antigen-specific, host-tolerant population of mature T cells. This process involves cellular proliferation, gene rearrangement, apoptotic cell death, receptor up- and downregulation and antigen-presentation processes, and is very vulnerable to a number of chemicals. Drugs may target different stages of T-cell differentiation like naive T cells, proliferating and differentiating thymocytes, antigen-presenting thymic epithelial cells and dendritic cells, cell death processes, etc. (Vos et al., 1999). The CWA may cause a depletion of peripheral T cells, particularly after prolonged treatment and during early stages of life when thymus activity is high and important in establishing a mature T-cell population. In addition, suppression of T cells may result in suppression of the adaptive immune system by affecting the maturation of B cells and thus antibody level.

42.3.3 Effects on initiation of immune responses

In response to the attacking pathogen, B and T lymphocytes generate antibodies and effector T cells that specifically recognize and neutralize or eliminate the invaders. The innate and adaptive immune systems act together to eliminate invading pathogens. Ideally, T cells tailor the responses to neutralize invaders with minimal damage to the host. The recognition of autoantigens is maintained by

the two distinct signals that govern lymphocyte activation. One is the specific recognition of antigen via clonally distributed antigen receptors and the other is antigen-nonspecific costimulation or "help" and involves interactions of various adhesive and signaling molecules expressed in response to tissue damage, linking initiation of immune responses to situations of acute "danger" for the host (Vos et al., 1999). This helps to aim immune responses at potentially dangerous microorganisms (nonself), while minimizing deleterious reactions to the host (self). Xenobiotics, however, can interfere with the initiation of immune responses if they act as antigens, by forming haptens or by releasing previously hidden self-antigens. They may also trigger an inflammatory response, or disturb T–B-cell cooperation.

CWAs with large molecular weight can function as antigens and become targets of specific immune responses themselves. This is particularly relevant for foreign protein pharmaceuticals, as these can activate both T and B lymphocytes. The resulting immune responses may lead to formation of antibodies, and induce specific memory which can lead to allergic responses to the drug. Immunotoxic effects may occur after repeated treatment with the same CWA. However, low-molecular-weight CWAs cannot function as antigens, because they are too small to be detected by T cells. Reactive chemicals that bind to proteins, however, can function as haptens and become immunogenic if epitopes derived from them prime T cells, which in turn provide costimulation for hapten-specific B cells. This effect is responsible for allergic responses to many new (neo) epitopes formed by chemical haptens.

Modification of autoantigens can also lead to autoimmune responses to unmodified self-epitopes. Haptenated autoantigens can be recognized and internalized by antigen-presenting cells. These cells subsequently present a mixture of neo- and self-epitopes complexed to distinct class II major histocompatibility (MHC-II) molecules on their surface and neospecific T cells. Th cells provide signals for the B cell. This leads to production of either antihapten or antiself antibodies depending on the exact specificity of the B cell. Moreover, once these B cells are activated, they can stimulate autoreactive Th cells recognizing unmodified self-epitopes. This process is called epitope (determinant) spreading and causes the diversification of adaptive immune responses. For example, injection of mercury salts initially induces a response directed only to unidentified chemically created neoepitopes, but after 3–4 weeks includes reactivity to unmodified self-epitopes. Thus the allergic response may gradually culminate as autoimmune responses reflecting the relative antigenicity of the neo- and self-epitopes involved (Lu and Kacew, 2002).

42.3.4 Induction of inflammation and noncognate T–B cooperation

Exposure to the CWAs is associated with an immune and inflammatory pathway mediating the release of proinflammatory cytokines like tumor necrosis factor α (TNFα), interleukin-1 (IL-1), IL-6, and attracts inflammatory cells like granulocytes and macrophages. Cytokines produced during this inflammatory response activate antigen-presenting cells and accumulation of tissue debris. The epitopes of antigens on debris provide costimulation for Th cells, which lead to the initiation of an adaptive immune response. Reactive xenobiotics may also stimulate adaptive immune responses by disturbing the normal cooperation of Th and B cells. Normally, B cells receive stimulation from Th cells that recognize (epitopes of) the same antigen. However, when Th cells respond to nonself-epitopes on B cells, such B cells may be noncognately stimulated by the Th cell. This occurs during graft-versus-host responses following bone marrow transplantation, when Th cells of the host recognize nonself-epitopes on B cells of the graft and vice versa. Drug/chemical-related lupus is characterized by a similar spectrum of autoantibodies which leads to T- and B-cell activation and results in production of autoantibodies to distinct autoantigens like DNA, nucleoli, nuclear proteins, erythrocytes, and basal membranes.

42.4 Exposition of autoantigens and interference with co-stimulatory signals

Co-stimulatory molecules expressed on the surface of various cells play a decisive role in the initiation and sustenance of immunity. Self-tolerance involves specific recognition of autoantigen leading to selective inactivation of autoreactive lymphocytes at birth, but tolerance is not established for (epitopes of) autoantigens that are normally not available for immune recognition. Pharmaceuticals can expose such sequestered epitopes by disrupting barriers between the antigen and the immune system (i.e., blood–brain barrier, blood–testis barrier, cell membranes). Tissue damage, cell death, and protein denaturation induced by chemicals can largely increase the chances of such (epitopes of) autoantigens for immune recognition. Antigen recognition followed by costimulation of signaling molecules leads to activation of lymphocytes and initiation of immune responses. Many xenobiotics have the inherent capacity to induce or inhibit this costimulation due to their intrinsic adjuvant activity. An array of co-stimulatory molecules is displayed on the surface of APCs and T cells. The level of the expression of the co-stimulatory molecules may play an important role during the course of acute disease and its remission

or relapse. Hence, modulation of these molecules may help to develop immunotherapeutic strategies (Khan et al., 2012).

42.5 Regulation of the immune response

The immune response elicited in response to a foreign pathogen or allergen is the result of a complex interplay of cytokines produced by macrophages, dendritic cells, mast cells, granulocytes, and lymphocytes. Immunotoxic chemicals can lead to either immunosuppression or immune exaggeration, that is, hypersensitivity and autoimmunity. Hypersensitivity is an immune response that damages the body's own tissues. Hypersensitivity reactions require a presensitized (immune) state of the host. They are divided into four classes (types I–IV) based on the mechanisms involved and the time course of the hypersensitive reaction. Type I hypersensitivity is an immediate or anaphylactic reaction, often associated with allergy. Symptoms can range from mild discomfort to death. Type I hypersensitivity is mediated by IgE released from mast cells and basophils. Type II hypersensitivity occurs when antibodies bind to antigens on the patient's own cells, marking them for destruction. This is also called antibody-dependent (or cytotoxic) hypersensitivity, and is mediated by IgG and IgM antibodies. Immune complexes (aggregations of antigens, complement proteins, and IgG and IgM antibodies) deposited in various tissues trigger type III hypersensitivity reactions. Type IV hypersensitivity (also known as cell-mediated or delayed-type hypersensitivity) usually takes between 2 and 3 days to develop. Type IV reactions are involved in many autoimmune and infectious diseases, but may also involve contact dermatitis (poison ivy). These reactions are mediated by T cells, monocytes, and macrophages. Actual development of clinical symptoms is influenced by the route and duration of exposure, the dosage of the pharmaceutical, and by immunogenetic (MHC haplotype, Th1-type vs Th2-type responders) and pharmacogenetic (acetylator phenotype, sulfoxidizer, Ah receptor, etc.) predisposition of the exposed individual. Moreover, atopic individuals that tend to mount Th2 immune responses are more susceptible to anaphylaxis triggered by an IgE response to chemical haptens than typical Th1 responders.

The dysregulation of the immune system can be characterized in several ways. Immune dysregulation can also be in the form of immune suppression and both innate and adaptive arms of the immune systems play crucial roles. A wide variety of physiological, pharmacological, and environmental factors can exert a negative influence on the immune system and sometimes result in immunotoxicity. Experimental data have shown that emotional and environmental stressors influence the functioning of the immune system and this is reflected in the various markers of specific immunity (Ray et al., 1991; Koner et al., 1998). Such experimental stressors consistently suppressed both humoral and cell-mediated immune (CMI) responses in experimental animals. Both antibody-forming cell counts and antibody titer were lowered and a neuroendocrine–immune axis concept was proposed. Similar attenuations in CMI responses were also seen after such stressors and DTH responses, leukocyte/macrophage migration indices, and also cytokine profiles (both Th1 and Th2 dependent). Further analysis of the mechanisms involved indicated that CNS-mediated changes could have contributed to this immunotoxicity. Depletion or antagonism of brain dopamine aggravated emotional stress-induced immune suppression, whereas psychoactive drugs like benzodiazepines and opioids prevented this response (Ray et al., 1992; Puri et al., 1994). In another set of experiments, rats exposed to several environmental pollutants like DDT showed graded degrees of immune suppression and immunotoxicity, when the exposure lasted for a reasonably long period of time. Gradual accumulation in the various body tissues resulted in a variety of untoward effects in the immune system, which was particularly susceptible to such xenobiotic-induced damage (Banerjee et al., 1996; Koner et al., 1998). Both humoral and CMI responses were affected, depending on the quantum and duration of exposure to these xenobiotics. Further, a combination of emotional stress and xenobiotic exposure had additive effects on the immunotoxicity parameters studied (Banerjee et al., 1997). Recent studies revealed that such emotional stress and xenobiotic-induced immunotoxicity was accompanied by derangements in oxidative stress parameters, such as enhancements in MDA levels and lowering of GSH/SOD levels in the blood (Koner et al., 1997; Ray and Gulati, 2007; Gulati et al., 2007; Ray et al., 2015; Thakur et al., 2017).

42.6 Immunotoxicity of chemical warfare agents

Xenobiotic-induced hypersensitivity reactions and autoimmune disorders are a major concern, whereas some of CWAs result in immunosuppression. Very few studies have been conducted to explore the immunomodulation and immunotoxic potential of CWAs, and there is little evidence that these drugs are associated with such undesirable, immunologically significant effects. The reason may be due to confounding factors such as stress, nutritional status, lifestyle, comedication, and genetics (Vos et al., 1999). Few conventional compounds have been shown to induce unexpected enhancement of immune competence. In particular, impaired activity of the first line of defense of the natural immune system can have

disastrous consequences. These are generally not influenced by the genetic predisposition of the exposed individual, but on actual outbreak of infections and the general immune status prior to exposition. This explains why immunosuppressive xenobiotics are most likely to have clinical consequences in immunocompromised individuals such as young children, the elderly, and can be aggravated further by stressful situations. Thus, the conduct of immunotoxicity studies on such CWAs is important to understand the mechanisms underlying these effects in a biological system.

There are thousands of toxic substances, but only a few are considered CWAs based on their characteristics, such as high toxicity, rapid action, and persistency. The exposure to CWA, depending on the type of agent and duration of exposure can result in immunodepressed conditions on the one hand, to allergic and autoimmune diseases on the other. CWAs can be classified in many different ways. Chemical warfare (CW) agents may be encountered as solids, liquids, or gases. Volatile substances may mainly contaminate the air and persistent substances, which are nonvolatile, mainly cover surfaces. Some agents, for example, sulfur mustard, may appear as solids under North European winter conditions (freezing point 14.4°C), as a liquid at a wide range of temperatures (boiling point 219°C), or as a vapor evaporating from the liquid phase. CW agents may also be encountered as mixtures or solutions of one agent in another, or of an agent in a solvent. The mixing of lewisite with sulfur mustard has been undertaken to lower the vapor pressure and freezing point of the mustard and hence to increase its persistence, without reducing the effective CW payload of weapon systems.

CWAs mainly used against people may also be divided into lethal and incapacitating categories. A substance is classified as an incapacitating agent if less than 1/100th of the lethal dose causes incapacitation, for example, through nausea or visual problems. The limit between lethal and incapacitating substances is not absolute but refers to a statistical average. CWAs are generally classified according to their principal target organs.

1. *Nerve agents.* These agents are extremely toxic compounds that work by interfering with the nervous system, and include soman, sarin, cyclosarin, tabun, and VX.
2. *Blister agents/vesicants.* These compounds severely blister the eyes, respiratory tract, and skin on exposure, and include nitrogen mustard, sulfur mustard, lewisite, etc.
3. *Choking agents.* These agents cause severe irritation, primarily affecting the respiratory tract, and include phosgene, ammonia, methyl bromide, methyl isocyanate, etc.
4. *Blood agents.* These agents are absorbed into the blood and interfere with the oxygen-carrying capacity, for example, arsine, cyanides, carbon monoxide, etc.

42.6.1 Nerve agents

Terroristic attacks in Japan in 1994 and 1995 (Ohtomi et al., 1996) proved that nerve agents are highly toxic organophosphorus compounds (OPs) and deadliest of CWAs, which represent potential threats to both military and civilian populations. These agents have both chemical names as well as two-letter NATO codes. These are categorized as G series agents: GA (tabun), GB (sarin), GD (soman), GF (cyclosarin), and V series agents: VE, VG, VM, and VX, the letter "G" representing the country of origin "Germany" and the letter "V" possibly denoting "venomous." Their initial effects occur within 1−10 min of exposure, followed by death within 15−30 min for sarin, soman, and VX, and within 30−60 min for tabun.

On the basis of scientific literature published on health effects of exposure to OP nerve agents and insecticide nerve agents in humans and animal studies, their short- and long-term effects are compiled. Four distinct health effects are identified: acute cholinergic toxicity; OP-induced delayed neuropathy (OPIDN); subtle long-term neuropsychological and neurophysiological effects; and a reversible muscular weakness called "intermediate syndrome." Each effect has data suggesting threshold exposure levels below which it is unlikely to be clinically detectable. High-level exposure results in definitive cholinergic poisoning; intermediate-level threshold cholinergic effects include miosis, rhinorrhea, or clinically measurable depression of cholinesterase; and low-level exposure results in no immediate clinical signs or symptoms. However, in combat settings, such as Iraq and Afghanistan, mild traumatic brain injury (mTBI) has been reported with lower level exposures or subclinical exposure (Gregory et al., 2014). Mohamed et al. (2016) reviewed that exposure to sarin in animal models resulted in neurotoxicity that approximates human mTBI in subclinical exposure. Animal studies show that low-level blast pressure waves are transmitted to the brain, thus causing behavioral, biochemical, pathological, and physiological effects on the nervous system. Further, Gregory et al. (2014) concluded that low-level blasts have long-term effects on the nervous system. Threshold exposure levels for known long-term effects of OP nerve agent are above intermediate-level exposure (Brown and Brix, 1998).

Sarin (GB, O-isopropyl methylphosphonofluoridate) is a potent OP nerve agent that irreversibly inhibits AChE. The ease and low cost of production make sarin gas a tool of mass destruction in the hands of terrorist groups and rogue nations. While people in the immediate vicinity of

a sarin attack may receive neurotoxic doses, people remote from the vicinity are likely to receive subclinical exposures. The subsequent build-up of acetylcholine (ACh) in the CNS causes seizures and respiratory arrest and at peripheral autonomic synapses causes "cholinergic crisis" due to accumulation of ACh. At high doses, exposure to sarin can cause tremors, seizures, and hypothermia. More seriously, accumulation of ACh at neuromuscular junctions (NMJs) also can cause paralysis and ultimately peripherally mediated respiratory arrest which can lead to death. Apart from its primary action on the cholinergic system, sarin can cause other indirect effects such as activation of several neurotransmitters including gamma-amino-butyric acid (GABA) and the alteration of other signaling systems such as ion channels, cell adhesion molecules, and inflammatory regulators. Moreover, sarin exposure is associated with toxic and immunotoxic effects (Mohamed et al., 2016). Subclinical doses of sarin cause subtle changes in the brain, and subclinical exposure to sarin has been proposed as an etiology to the Gulf War syndrome. Gulf War illness (GWI) is a chronic multisymptom disorder affecting veterans of the 1990−91 Gulf War. GWI was linked with exposure to chemicals including the nerve gas prophylactic drug pyridostigmine bromide (PB) and pesticides (DEET, permethrin). Veterans with GWI exhibited prolonged, low-level systemic inflammation. Anca et al. (2018) assessed the effects of GWI-related chemicals on macrophage infiltration and its subsequent influence on hepatic cholestasis using Sprague-Dawley rats. Exposure to GWI-related chemicals alone increased IL-6, CD11b + F4/80 − macrophages and developed worse liver pathology due to sustained low-level inflammation of the liver when compared to animals without GWI.

The wide use of cholinesterase inhibitors in various spheres of human life and the risk of acute and chronic intoxications associated with this process prompted investigation of the role of acetylcholinesterase (AChE) and nonspecific esterases in the immunotropic effects of these chemicals. They irreversibly bind to AChE that normally catalyzes the hydrolysis of ACh at the cholinergic synapses and NMJs. The inhibition of degradation results in accumulation of ACh in the cholinergic synapses, causes the overstimulation of peripheral as well as central cholinergic nervous systems, and is clinically manifested as acute cholinergic crisis (diarrhea, sweating, salivation, miosis, convulsions, respiratory failure, and/or death) (Marrs, 1993; Taylor, 2006).

Exposures to seizure-inducing chemical threat agents such as OP cholinesterase inhibitor, diisopropylphosphorofluoridate (DFP), and the GABA receptor inhibitor, tetramethylenedisulfotetramine (TETS), also caused convulsions and long-term neurological sequelae in survivors, and represent a major public health concern (Isaac et al., 2016). The toxic organic compound, TETS, is used as an effective rodenticide, unfortunately, human poisoning by this substance occurs commonly. Every year, the largest number of poisonings is reported in China and dozens of poisonings happen annually in the United States. TETS is one of the most hazardous pesticides and also a possible CWA with no known antidote (Patocka et al., 2018).

Phosphine (PH3) inhalation is characterized by a steep probit slope of 11, ranking it among CW nerve agents such as sarin, which has a probit slope in the 9.4−13.2 range. It is another toxidrome-spanning chemical that is widely used as an insecticide and rodenticide. Exposure to PH3 can cause a host of target organ and systemic effects, such as oxidative stress, cardiopulmonary toxicity, seizure-like activity, and overall metabolic disturbance, altogether leading to death (Benjamin et al., 2017).

42.6.1.1 Immunotoxicity of nerve agents

Low doses of sarin are shown to be highly immunosuppressive and reduce glucocorticoid production (Kalra et al., 2002). The effects of sarin exposure on the immune system are attenuated by ganglionic blockers and decreased glucocorticoid level may be a biomarker for cholinergic toxicity. In addition, nerve agents cause the activation of multiple noncholinergic neurotransmitter systems in the central nervous system (CNS), thus resulting in mutagenic, stressogenic, immunotoxic, hepatotoxic, membrane, and hematotoxic effects (Bajgar, 1992). The CNS and the immune system communicate bidirectionally, and cholinergic agents modulate the immune system. The ability of OP compounds to induce an alteration of the immune system was primarily demonstrated in animals or humans exposed to OP insecticides (OPIs). The results provide evidence that, especially neutrophil function, natural killer cell, cytotoxic T-cell, and humoral immune functions, and spontaneous as well as mitogen-induced lymphocyte proliferation, are altered in animals or humans exposed to OP compounds (Li et al., 2002; Newcombe and Esa, 1992). In addition, a decreased number of cells in the spleen and thymus (Ladics et al., 1994), an inhibition of chemotaxis in neutrophils, inhibition of monocyte accessory functions, or inhibition of interleukin-2 production (Casale et al., 1993; Pruett and Chambers, 1988) were reported following exposure to OPs, at relatively high toxic doses.

Lee et al. (1979) were the first to demonstrate that lymphocyte proliferation was decreased in the presence of OPs. Although most of the studies described the results of OPI exposure, there are studies about the immunotoxic effects of highly toxic nerve agents and their byproducts. Marked impairment in neutrophil chemotaxis and neutrophil adhesion and a reduction in the natural killer cell and

cytotoxic T-cell function were observed in workers exposed to OPIs and byproducts of sarin (Newcombe and Esa, 1992; Li et al., 2002). Kant et al. (1991) documented a decrease in the weight of thymus, an important immune organ in severely affected soman survivors, but other tests of immune function did not show differences between control and soman-exposed rats. Samnaliev et al. (1996) described a decrease in the number of plaque-forming cells in soman-exposed rats after the administration of sheep red blood cells (SRBCs) as an antigen. However, Johnson et al. (2002) demonstrated that OP-induced modulation of immune functions can involve not only their suppression but also their activation. Similar activation of some immune functions involving an "acute phase response," such as an increase in the synthesis of acute phase proteins, increase in release of histamine from basophile leukocytes, and activation of macrophages were observed following exposure to soman (Sevaljevic et al., 1992; Newball et al., 1986). Although most of the studies dealt with exposure to high doses, Kassa et al. (2004) confirmed that not only symptomatic but also asymptomatic doses of nerve agent sarin were able to modify various immune functions. The proportion of T lymphocytes was found to be decreased, while the B-cell levels were raised. However, sarin significantly suppressed nonspecific in vitro stimulated proliferation of both T and B cells, which suggests that it can also block the normal immune response to infection. Immunosuppression may result from direct action of ACh upon the immune system or it may be secondary to the toxic chemical stress associated with cholinergic poisoning (Pruett et al., 1992). Further, immunomodulation at low levels seems to be very complex and it is suggested that there are probably other protein targets very sensitive to some anticholinesterases, including nerve agents. However, the function of these protein targets is not yet known (Ray, 1998). Some immune functions are probably stimulated due to the development of "acute phase response" generally characterized for inflammatory reaction of OP-exposed organisms (Sevaljevic et al., 1989, 1992). Other immune functions are suppressed due to the immunotoxicity of OP compounds. Although these findings are difficult to extrapolate directly to low-level exposures to nerve agents, they indicate that subtle alteration of the immune system could also occur in humans at exposure levels which do not cause any clinical manifestation. Postintoxication immunodeficiency can promote infectious complications and diseases.

AChE has been shown to be located on the plasma membrane of T lymphocytes, while B cells are esterase negative (Szelenyi et al., 1982). Thus, AChE inhibition by toxic agents in sublethal doses may play an important role in immunodeficiency following exposure to nerve gases. Zabrodskii et al. (2003) showed inhibition of AChE in T cells and the decrease in the number of esterase-positive T lymphocytes (and, to a certain extent, in monocytes and macrophages) directly correlated with suppression of T-cell-dependent antibody production and to the degree of DTH reduction, on exposure to dimethyl dichlorovinyl phosphate, sarin, VX, lewisite, tetraethyl lead, and dichloroethane. This presumably involves the loss of some functions by T lymphocytes (e.g., by Th1 cells), which leads to attenuation of T-dependent immune reactions. Thus, the anticholinesterase effect of lewisite, TEL, and DCE may be one of the important mechanisms in the formation of T-cell-mediated immunodeficiency. A study showed that malathion (an analog of VX) in acute noncholinergic doses enhanced the humoral immune response to SRBCs, and macrophage function, and also caused mast cell degranulation. When effects of acute administration of malathion were observed in mast cell-deficient mice, the humoral activation was not observed, thus suggesting that the mast cells contribute to the increases in macrophage function and humoral immunity observed in normal mice (Rodgers et al., 1996).

The effects of subchronic doses of malathion exposure on humoral and CMI responses were studied in male albino mice, rats, and rabbits using SRBCs, tetanus toxoid and ovalbumin as antigens. Malathion resulted in (1) attenuation in antigen-induced antibody response, (2) suppression of PFC, and (3) marked inhibition of LMI and MMI factors. Subchronic malathion exposure induced differential degrees of humoral and CMI suppression in these experimental animals. However, both cellular and humoral immune responses were decreased in a dose−time-dependent pattern and a consistent trend was observed. Banerjee et al. (1998) reported that the threshold level of malathion for inducing immune suppression varies on the basis of species of animals, type of antigen used, and the method of immunological assay. In another study, rats were administered with malathion alone and in combination with bradykinin potentiating factor (BPF), and effects were compared to a vehicle group on immune parameters. The results showed that the concentration of total globulin, total immunoglobulins, IgG, IgM, circulatory immune complexes, total number of RBC and platelets, and hemoglobin concentrations decreased significantly in malathion-exposed animals. The results suggested that exposure to malathion has negative effects on immune function that were mediated through alteration of cytokines, antioxidants, and direct damage of BM. Also, BPF can ameliorate both physiological and morphological changes (Ahmed, 2012). In another human study involving malathion-poisoned subjects, high levels of malathion were associated with significant enhancements of IL-2, IL-4, and TNF-α levels in blood, whereas no significant changes in immunoglobulin levels were seen. This study thus showed altered levels of cytokines in the

blood of malathion-exposed subjects (Seth et al., 2008). Kassa et al. (2001) showed that rats exposed once or repeatedly to three various low concentrations of sarin for 60 min in an inhalation chamber induced immunotoxicity. Nonconvulsive concentrations of sarin caused subtle suppression of spontaneous, as well as lipopolysaccharide-stimulated, proliferation of spleen lymphocytes and the bactericidal activity of peritoneal macrophages.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a well-known explosive compound causing neurotoxicity. In an experimental study by Youping et al. (2014), miRNA and mRNA were used as novel biomarkers to study the molecular mechanisms for RDX-induced neurological disorder and neurotoxicity. In their study, it was found that immune and inflammation response genes are highly enriched in differentially expressed mRNAs after exposure to RDX. Interestingly, the immune and inflammation response genes connected network indicated that RDX could first modulate miRNA expression and then trigger an immune and inflammation gene network. For instance, miR-98 could target IL10, which then upregulates TNF, which induces the expression of the transcription factor HSF1, to carry out immune functions. Their findings on RDX-regulated immune and inflammation responses could contribute to RDX-induced neurotoxicity.

42.6.2 Blister or vesicant agents

Blister agents act on skin and other epithelial tissues and severely blister the eyes, respiratory tract, and internal organs, and also destroy different substances within cells of living tissue. The symptoms are variable depending upon the compound and the sensitivity of the individual. Acute mortality is low; however, they can incapacitate the enemy and overload the already burdened healthcare services during war time. Some of these agents are SM (sulfur mustard), HN (nitrogen mustard), L (lewisite), and CX (phosgene oximine).

Sulfur mustard was the most widely used CWA in the Iran–Iraq War, resulting in over 100,000 chemical casualties between 1980 and 1988. It acts as an alkylating agent with long-term toxic effects on several body organs, mainly the skin, eyes, and respiratory system. The extent of tissue injury depends on the duration and intensity of exposure. When absorbed in large amounts, SM can damage rapidly proliferating cells of bone marrow and may cause severe suppression of the immune system. In the pulmonary system, epithelial cells and macrophages are the primary layer of cells that can be exposed to SM. Following SM exposure, intense cellular and molecular modifications occur in the normal cells of lung. During several days after exposure, innate immunity induces an adaptive immune system with proinflammatory mediators. If the apoptosis and necrosis rate increase, cell contents are released into the extracellular matrix (ECM), and immune cells are activated. Epithelial cell detachment, cell death, fibrosis, DNA repair system activation, tissue repair induction, and systemic signaling have been reported after SM exposure (Imani et al., 2015).

42.6.2.1 Immunotoxicity of blister agents

Several lines of investigation have provided evidence that SM causes immunosuppression in humans. The earliest evidence came from clinical observations of humans directly exposed to sulfur mustard during World War I, who showed significant changes (quantitative and qualitative) in the circulating elements of the immune system. Stewart (1918) studied 10 fatal cases of mustard poisoning and observed striking depression of bone marrow production of WBCs. Among the sulfur mustard casualties during the Iran–Iraq conflict, leukopenia was accompanied by total bone marrow aplasia and extensive losses of myeloid stem cells, and proved an association between suppression of immunologic functions and an increased incidence of infectious disease (Eisenmenger et al., 1991).

SM was widely used during the Iran–Iraq conflict and there are many reports of the influence of SM on the respiratory, gastrointestinal, and endocrine systems, as well as the immune system (Balali-Mood and Farhoodi, 1990; Emad and Razaian, 1997; Sasser et al., 1996; Budiansky, 1984). The influence of SM on the immune system has been the subject of many researches since 1919 (Krumbhaar and Krumbhaar, 1919). Early investigations on SM casualties during the Iran–Iraq War showed decreased immunoresponsiveness, expressed as leukopenia, lymphopenia, and neutropenia, as well as hypoplasia and atrophy of the bone marrow (Tabarestani et al., 1990; Balali-Mood et al., 1991). Chronic exposure to SM has been associated with the impairment of NK cells among workers of poison gas factories in Japan (Yokogama, 1993). Similarly, cell-mediated immunity was found to be suppressed following mustard gas exposure (Zandieh et al., 1990).

Leukopenia is the first manifestation to appear within the first few days postexposure. Thrombocytopenia and anemia followed later if the patients survived (WBC counts of some patients dropped to less than 1000 per cm^3). Although most of these patients suffered skin burns, clinicians reported cases that had minor skin lesions and yet developed leukopenia. Bone marrow biopsies revealed hypocellular marrow and cellular atrophy involving all elements. Studies on the status of immunocompetent cells in the blood of patients exposed to sulfur mustard showed that T-cell and monocyte counts dropped in 54% and 65% of the patients, respectively, from day 1 and up to the seventh week postexposure (Hassan and Ebtekar, 2002). The majority of the patients showed increased

levels of IgG and IgM during the first week, but the percentage decreased over the next 6 months. The percentage of patients with increased levels of C3, C4, and CH50 was somewhat higher than of healthy controls during the first week and up to the sixth month (Tabarestani et al., 1990) and remained higher 3 years postexposure, especially in the severely affected group. Eight years after exposure there was a significant increase in the number of atypical leukocytes (such as myelocytes). The severely affected group presented with significantly lower CD56 NK, as well as CD4 and CD8 counts, compared with healthy controls (Yokogama, 1993). However, Hassan and Ebtekar (2002) reported that there was no major difference between the severely affected patients and healthy controls concerning CD19 B cells, CD14 monocytes, and CD15 granulocytes. The moderately and mildly affected patients did not significantly differ in their leukocyte subset counts from the control group 8 years after exposure (Mahmoudi et al., 2005). Follow-up studies on the clinical conditions of exposed Iranian victims still show that they suffer from three major problems: recurrent infection, septicemia and death, respiratory difficulties and lung fibrosis, as well as a high incidence of malignancies, septicemia, and death. Hassan and Ebtekar (2002) suggested that patients with moderate clinical manifestations may be experiencing a shift from Th1 to Th2 cytokine patterns since leukocyte cultures from this patient group showed a decrease in IFN-γ levels.

When absorbed in large amounts, SM can damage rapidly proliferating cells of bone marrow and may cause severe suppression of the immune system (Sasser et al., 1996). Moreover, this alkylating agent has been reported to produce short- and long-term suppression of antibody production in both animals and humans. It also affects complement system factors C3 and C4. Incidences of acute myelocytic and lymphocytic leukemia are reported to be 18 and 12 times higher in patients exposed to SM, in comparison with the normal group, respectively (Zakeripanah, 1991). reported that exposure to SM could result in the impairment of human immune function, especially in the number of B and T lymphocytes. Hence, SM is still a potential threat to the world and effective therapeutic measures must be taken for the relief of the victims of this incapacitating agent. Ghotbi and Hassan (2002) showed that the percentage of NK cells, playing an important role in cellular immunity, was significantly lower in severe patients than in the control group. Studies on animal models have shown that alkylating agents such as SM mainly affect B cells, which is why hypogammaglobulinemia is one of the main features in animal models, whereas studies on human cases, following treatment with cytotoxic drugs, suggest that low-dose exposure to alkylating agents impairs cellular immunity and high-dose exposure to such agents impairs both cellular and humoral responses (Marzban, 1989; Malaekeh et al., 1991). There are reports suggesting that sulfur mustard can produce toxicity through the formation of reactive electrophobic intermediates, which in turn covalently modify nucleophilic groups in biomolecules such as DNA, RNA, and protein (Malaekeh et al., 1991). As a result, these agents are particularly toxic to rapidly proliferating cells including neoplastic, lymphoid, and bone marrow cells. Mahmoudi et al. (2005) reported higher IgM levels after 16−20 years of exposure to SM, compared to the control group. A significant decrease in the number of NK cells in severely affected patients is probably due to the destructive effect of this alkylating agent on NK cell precursors in bone marrow. However, activity of NK cells was found to be noticeably above normal, which possibly compensates for the reduction in the number of these cells.

Korkmaz et al. (2006) explained the toxicodynamics of sulfur mustards in three steps: (1) binding to cell surface receptors; (2) activation of ROS and RNS leading to peroxynitrite ($OONO^-$) production, and (3) $OONO^-$-induced damage to lipids, proteins, and DNA, leading to polyadenosine diphosphate ribose (PARP) activation. This could provide a lead for devising strategies for protection against/treatment of mustard toxicity.

A study has been conducted to evaluate the incidence of immunocompetence, among survivors of the chemical bombardment of Halabja in the Kurdistan region of Iraq. The result revealed that there was a deficiency in antibody-mediated immunity. There were significant differences between the exposed and the control samples with respect to total leukocytes, neutrophil count, lymphocyte count, IgG, and IgA. The immunological reactions were more closely related to the effects of mustard gas, which appeared to be long-lasting. They concluded that long-term effects were produced by CWAs on victims who have survived in Halabja and, in particular, on their immune system at both antibody- and cell-mediated levels. This study confirmed the immunosuppressive property of mustard compounds, and indicates increased vulnerability of the exposed individuals to secondary opportunistic or pyogenic bacterial infections due to injuries which occurred frequently among chemical survivors in Halabja (Hama et al., 2008).

In another study, Kumar et al. (2015) showed that topical application of nitrogen mustard (NM), an analog of sulfur mustard (SM), on mice caused an increased in the expression of inflammatory mediators, microvesication, and apoptotic cell death. Further, NM also induced the activation of MAPKs/ERK1/2, JNK1/2, and p38 as well as that of Akt together with the activation of transcription factor AP1. NM exposure caused a very strong and time-dependent increase in COX-2 and iNOS levels in NM-exposed skin tissue compared to vehicle control. NM

exposure caused a 21-fold induction in iNOS levels at 24 h postexposure, which decreased thereafter at 72 and 120 h postexposure. The expression of TNF-α, a cytokine involved in inflammation, apoptosis, and immune system development also increased in a biphasic manner, where a time-dependent increase compared to control was observed up to 24 h, followed by a decline at 72 h and again an increase at 120 h post-NM exposure. Gelatinases, especially MMPs that could come from infiltrating neutrophils, have the ability to degrade basement membrane components and interrupt the epidermal−dermal junction. These also play an important role in vesicant-related inflammatory and immune responses.

Liemin et al. (2015) conducted a study to show that toxicity resulting from topical mustard exposure is mediated in part by initiating exaggerated host innate immune responses. They used an experimental model of skin exposure to NM and observed the activation of inflammatory dermal macrophages that exacerbate local tissue damage in an inducible nitric oxide synthase (iNOS)-dependent manner. Intervention with a single dose of 25-hydroxyvitamin D3 [25(OH)D] suppressed macrophage-mediated iNOS production, resulting in mitigation of local skin destruction, enhanced tissue repair, protection from marrow depletion, and rescue from severe precipitous wasting. These protective effects were confirmed using pharmacological inhibitors of iNOS. The study proposed the role of the host innate immune system in exacerbating injury following exposure to NM and supported the translation of 25(OH)D in therapeutic use against these chemical agents.

In another study, Alessandro et al. (2015) reported persistent pulmonary injury progressing to fibrosis, accompanied by a macrophage inflammatory response following NM exposure. The appearance of M1 and M2 macrophages in the lung correlated with NM-induced acute injury and the development of fibrosis, suggesting a potential role of these macrophage subpopulations in the pathogenic response to NM. Thus their study demonstrated that proinflammatory/cytotoxic (M1) and antiinflammatory/profibrotic (M2) macrophages accumulate sequentially in the lung after nitrogen mustard exposure. Therefore, for clinical relevance, their study suggested that targeting these macrophage subsets may be useful as a novel therapeutic strategy against NMt-induced lung injury. Lewisite (LEW) is a potent arsenical vesicating CWA used as a terrorist weapon. Ocular tissue is exquisitely sensitive to LEW and exposure can cause devastating corneal lesions. Tewari-Singh et al. (2017) evaluated the pathophysiology of the corneal injury in rabbits following LEW vapor exposure and analyzed the expression of COX-2 (an inducible enzyme expressed in mononuclear phagocytes and neutrophils that is involved in proinflammatory prostaglandin synthesis) and changes in other inflammatory cytokines via cytokine array. Interestingly, LEW exposure caused an increase in the number of blood vessels and inflammatory cells. LEW also caused an increase in the expression levels of COX-2, IL-8, MMP-9, and VEGF, indicating their involvement in LEW-induced inflammation, vesication, and neovascularization.

In conclusion, the results suggest that exposure to blister agents causes a higher risk of opportunistic infections, septicemia, and death following severe suppression of the immune system, especially in the case of lesions and blisters produced by these agents. As alkylating agents, they form covalent linkages with biologically important molecules, resulting in disruption of cell function, especially cell division. As a result, these agents are particularly toxic to rapidly proliferating cells, including neoplastic, lymphoid, and bone marrow cells. However, there is still a paucity of information regarding the long-term immunosuppressive properties of alkylating agents in the setting of battlefield exposure to this agent.

42.6.3 Choking agents

Choking agents, such as phosgene (CG), diphosgene (DP), chlorine, and chloropicrin (PS), act on the pulmonary system causing severe irritation and swelling of the nose, throat, and lungs. These inhalational agents damage the respiratory tract and cause severe pulmonary edema in about 4 h, leading to death. The effects are variable, rapid, or delayed depending on the specific agent (Gift et al., 2008).

Phosgene was first used as a chemical weapon in World War I by Germany and later as an offensive capability by French, American, and British forces. In this conflict, phosgene was often combined with chlorine in liquid-filled shells, so it was difficult to state the number of casualties and deaths attributable solely to phosgene. In military publications, it has been referred to as a choking agent, pulmonary agent, or irritant gas. Since World War I, phosgene has rarely been used by traditional militaries, but the extremist cult Aum Shinrikyo used this agent in an attack against the Japanese journalist Shouko Egawa in 1994. Nowadays, phosgene is primarily used in the polyurethane industry for the production of polymeric isocyanates. Phosgene is also used in the polycarbonate industry and in the manufacture of carbamates and related pesticides, dyes, pharmaceuticals, and isocyanates. Suspected sources of atmospheric phosgene are fugitive emissions, thermal decomposition of chlorinated hydrocarbons, and photooxidation of chloroethylenes. Individuals are most likely to be exposed to phosgene in the workplace during its manufacture, handling, and use. Phosgene is extremely toxic by acute (short-term) inhalation exposure. Severe respiratory effects, including pulmonary edema, pulmonary emphysema, and death have been reported in

humans. Severe ocular irritation and dermal burns may result following eye or skin exposure. Chronic inhalation exposure to phosgene has been shown to result in some tolerance to the acute effects noted in humans, but may also cause irreversible pulmonary changes of emphysema and fibrosis (US Department of Health and Human Services, 1993).

Primarily because of phosgene's early use as a war gas, many exposure studies have been performed over the past 100 years to examine the effects and mode of action of phosgene following a single, acute (less than 24 h) exposure. Many studies have examined the effects of acute phosgene exposure in animals but the human data are limited to case studies following accidental exposures. Most studies were performed in rodents and dogs, with exposure concentrations ranging between 0.5 and 40 ppm (2−160 mg/m^3) and duration intervals ranging from 5 min to 8 h. Acute exposure studies in animals suggest that rodent species may be more susceptible to the edematous effects of phosgene acute exposure than larger species with lower respiratory volumes per body weight such as dogs and humans (Pauluhn, 2006; Pauluhn et al., 2007). Pauluhn et al. (2007) reported that acute phosgene exposure results in increased lung lavage protein, phospholipid content, enzyme levels, number of inflammatory cells, and lethality (LC_{50}). Rats seem to be able to survive approximately threefold higher levels of lung edema than humans (100- vs 30-fold), thus rat responses in short- and long-term assays may still be relevant to humans, even if it is ultimately shown that rats produce higher levels of edema following acute phosgene exposure.

42.6.3.1 Immunotoxicity of choking agents

Acute exposure to phosgene has been shown to result in immunosuppression in animals, as evidenced by an increased susceptibility to in vivo bacterial and tumor cell infections and viral infection as well as decreased in vitro virus-killing and T-cell response (Ehrlich and Burleson, 1991). Selgrade et al. (1989) reported that a single 4-h exposure to phosgene concentrations as low as 0.025 ppm significantly enhanced mortality due to streptococcal infection in mice. Later, Selgrade et al. (1995) administered *Streptococcus zooepidemicus* bacteria via an aerosol spray to the lungs of male Fischer-344 rats immediately after phosgene exposure and measured the subsequent clearance of bacteria. They showed that all phosgene concentrations from 0.1 to 0.5 ppm impaired resistance to bacterial infection and that the immune response is modulated by phosgene exposure. After 4 weeks following exposure, bacterial resistance as well as immune response returned to normal. Yang et al. (1995) also reported a decrease in bacterial clearance in the lungs at 24 h after infection following a single 6-h exposure to phosgene. Yang et al. (1995) also found that if the bacteria are administered 18 h after single phosgene exposures rather than immediately, the clearance is normal, which indicates that recovery from the toxic effect of phosgene is rapid. When inhaled, phosgene either is rapidly hydrolyzed to HCl and CO_2 and exhaled (Schneider and Diller, 1989) or penetrates deep into the lungs and is eliminated by rapid reactions with nucleophilic constituents of the alveolar region (Pauluhn et al., 2007).

As phosgene is electrophilic, it reacts with a wide variety of nucleophiles, including primary and secondary amines, hydroxy groups, and thiols. In addition, it also reacts with macromolecules, such as enzymes, proteins, or other polar phospholipids, resulting in a marked depletion of glutathione (Sciuto et al., 1996) and forms covalent adducts that can interfere with molecular functions. Phosgene interacts with biological molecules through two primary reactions: hydrolysis to hydrochloric acid and acylation reactions. Although the hydrolysis reaction does not contribute much to its clinical effects, the acylation reaction is mainly responsible for the irritant effects on mucous membranes. The acylation reactions occur between highly electrophilic carbon molecules in phosgene and amino, hydroxyl, and sulfhydryl groups on biological molecules. These reactions can result in membrane structural changes, protein denaturation, and depletion of lung glutathione. Acylation reactions with phosphatidylcholine are particularly important as it is a major constituent of pulmonary surfactant and lung tissue membranes. Exposure to phosgene has been shown to increases the alveolar leukotrienes, which are thought to be important mediators of phosgene toxicity to the alveolar−capillary interface. Phosgene exposure also increases lipid peroxidation and free radical formation. These processes may lead to increased arachidonic acid release and leukotriene production. Proinflammatory cytokines, such as interleukin-6, are also found to be substantially higher 4−8 h after phosgene exposure. In addition, studies have shown that postexposure phosphodiesterase activity increases, leading to decreased levels of cyclic AMP. Normal cAMP levels are believed to be important for maintenance of tight junctions between pulmonary endothelial cells and thus for prevention of vascular leakage into the interstitium. Oxygenation and ventilation both suffer, and breathing is dramatically increased.

Schneider and Diller (1989) reported that inhalation of phosgene at high concentrations results in a sequence of events, including an initial bioprotective phase, a symptom-free latent period, and a terminal phase characterized by pulmonary edema. The first is an immediate irritant reaction, likely caused by the hydrolysis of phosgene to hydrochloric acid on mucous membranes, which results in conjunctivitis, lacrimation, and oropharyngeal burning sensations. This symptom complex occurs only in

the presence of high-concentration (>3–4 ppm) exposures but does not have any prognostic value for the timing and severity of later respiratory symptoms. The most important finding to identify during this stage is a laryngeal irritant reaction causing laryngospasm, which may lead to sudden death. The irritant symptoms last only a few minutes and then resolve as long as further exposure to phosgene ceases. The second phase, when clinical signs and symptoms are generally lacking, may last for several hours after phosgene exposure. The duration of the latent phase is an extremely important prognostic factor for the severity of the ensuing pulmonary edema. Patients with a latent phase of less than 4 h have a poor prognosis. Increased physical activity may shorten the duration of the latent phase and worsen the overall clinical course. Unfortunately, there are no reliable physical examination findings during the latent phase to predict its duration. However, histologic examination reveals the beginnings of an edematous swelling, with exudation of blood plasma into the pulmonary interstitium and alveoli. This may result in damage to the alveolar type I cells and a rise in hematocrit. The length of this phase varies inversely with the inhaled dose. The third clinical phase peaks approximately 24 h after an acute exposure and if lethality does not occur, recedes over the next 3–5 days. In the third clinical phase of phosgene toxicity, the accumulating fluid in the lung results in edema. Oxygenation and ventilation both suffer, and the breathing is dramatically increased. Often, positive end expiratory pressure (PEEP) is required to stent open alveoli that would otherwise collapse and result in significant ventilation/perfusion (*V*/*Q*) mismatch. This hyperventilation causes the protein-rich fluid to take on a frothy consistency. A severe edema may result in an increased concentration of hemoglobin in the blood and congestion of the alveolar capillaries.

Increased levels of protein in bronchoalveolar lavage have been shown to be among the most sensitive endpoints characterizing the early, acute effects of phosgene exposure, and are rapidly reduced after the cessation of exposure (Sciuto, 1998; Sciuto et al., 2003). With continuous, chronic, low-level phosgene exposure, there may be transition of edema to persistent cellular inflammation, leading to the synthesis of abnormal type I collagen and pulmonary fibrosis. An increased synthesis of type I relative to type III collagen can lead to chronic fibrosis. Surfactant lipids are important for maintaining alveolar stability and for preventing pulmonary edema. Pauluhn et al. (2007) reported that the induction of surfactant abnormalities following phosgene exposures is a key pathophysiological event leading to pulmonary edema and chronic cellular inflammation, leading to the stimulation of fibroblasts and the synthesis of "abnormal" collagen in pulmonary fibrosis. Ehrlich and Burleson (1991) demonstrated an enhanced and prolonged viral infection using an influenza virus infectivity model in rats on days 3 and 4 following exposure to the toxicant gas phosgene. There are limited studies, in both humans and experimental animals, to evaluate immunotoxicity of chronic low-level environmental exposures to phosgene. The lack of studies examining the effects in humans or laboratory animals from chronic exposure to phosgene is a concern and the sequelae of effects leading to phosgene-induced pulmonary fibrosis is not well understood.

In a study, phosgene toxicity has been shown to stimulate the production of additional mediators of vasoconstriction (e.g., leukotrienes C4, D4, and E4) and suppress mediators of vasodilation (e.g., prostaglandins and cAMP), all of which can occur via ETA/ETB coupled signal transduction. Liberated arachidonic acid is then metabolized by lipoxygenase-producing leukotrines C4, D4, and E4. The release of these cysteinyl leukotrienes (LTC4, LTD4, and LTE4) leads to cellular contraction by stimulating the release of intracellular Ca^{2+} stores. Metabolism of AA can also result in the production of prostaglandins through cyclooxygenase activity, which in turn can stimulate cAMP synthesis inducing vasodilatation. These findings are in agreement with this proposed mechanism of phosgene toxicity, as there is no evidence of altered cyclooxygenase activity and levels of cAMP are diminished following phosgene exposure (Wesley et al., 2016).

Piotr et al. (2015), recently reported that phosgene exposure via an industrial or warfare release produces severe acute lung injury (ALI) with high mortality, characterized by massive pulmonary edema, disruption of epithelial tight junctions, surfactant dysfunction, and oxidative stress. There are no targeted treatments for phosgene-induced ALI. Nitric oxide synthase 2 (NOS-2) is upregulated in the lungs after phosgene exposure. They demonstrated that NOS-2 expression in lung epithelium exacerbates inhaled endotoxin-induced ALI in mice, mediated partially through downregulation of surfactant protein B expression. Therefore, they further conducted a selective NOS-2 inhibitor delivered to the lung epithelium by inhalation that mitigated phosgene-induced ALI. Inhaled phosgene produced increased lung NOS-2 expression at 24 h. Administration of aerosolized 1400W, a selective NOS-2 inhibitor, via inhalation significantly attenuated phosgene-induced ALI and preserved epithelial barrier integrity. They also demonstrated for the first time that NOS-2-derived nitric oxide downregulates the tight junction protein, that is, zonula occludens 1 expression at the transcriptional level in human lung epithelial cells, providing a novel target for ameliorating vascular leak in ALI. Their data demonstrated that lung NOS-2 plays a critical role in the development of phosgene-induced ALI and suggested that aerosolized NOS-2 inhibitors offer a novel therapeutic strategy for its treatment.

42.6.4 Blood agents

Blood agents like arsine, cyanide, and carbon monoxide are rapidly absorbed into the blood, affect its oxygen-carrying capacity, and produce seizures, respiratory failure, and cardiac arrest. Hydrogen cyanide has been known as a potent toxicant for over 200 years. It was used as a CWA during World War I by France. Although it is highly volatile (and was later considered "militarily useless" because of its volatility), no deaths from its military use during World War I were ever reported. After World War II, the importance of hydrogen cyanide as a CWA diminished rapidly, primarily as a result of the rise of nerve agents. Although reduced in importance, there are some reports of hydrogen cyanide being used as a war gas by Vietnamese forces in Thailand territories and during the Iran−Iraq War in the 1980s (Sidell, 1992). Hydrogen cyanide can be detoxified rapidly by humans. It is very volatile and massive amounts of the gas are needed for it to be effective as a CWA.

Cyanide is primarily an environmental contaminant of industrial processes. It is used in the metal-processing industry for electroplating, heat treating, and metal polishing and can be found in waste waters from many mining operations that use cyanide compounds in the extraction of metals, such as gold and silver, from ore. The acute toxicity of cyanide has been well documented in humans and experimental animals. Symptoms of toxicity in humans include headache, breathlessness, weakness, palpitations, nausea, giddiness, and tremors (Gupta et al., 1979). Depending on the degree of intoxication, symptoms may include "metallic" taste, anxiety and/or confusion, headache, vertigo, hyperpnea followed by dyspnea, convulsions, cyanosis, respiratory arrest, bradycardia, and cardiac arrest. Death results from respiratory arrest. Onset is usually rapid. Effects on inhalation of lethal amounts may be observed within 15 s, with death occurring in less than 10 min. Hydrogen cyanide should be suspected in terrorist incidents involving prompt fatalities, especially when the characteristic symptoms of nerve agent intoxication are absent. Chronic exposure to low-level cyanide can result in neuropathies, goiter, and diabetes. Cyanide and derivatives prevent the cells of the body from using oxygen. Cyanide acts by binding to mitochondrial cytochrome oxidase, blocking electron transport, thus inhibiting enzymes in the cytochrome oxidase chain and in turn blocking oxygen use in metabolizing cells and preventing the use of oxygen in cellular metabolism. These chemicals are highly toxic to cells and in high doses may result in death. Cyanide is more harmful to the heart and brain as these organs require large amounts of oxygen.

42.6.4.1 Immunotoxicity of blood agents

There are very few reports on immunotoxicity of blood agents; however, acrylonitrile (vinyl cyanide, VCN), an environmental pollutant which is metabolized to cyanide, has been shown to be an animal and human carcinogen, particularly for the gastrointestinal tract (Mostafa et al., 1999; National Toxicology Program Technical Report Series, 2001). Earlier, Hamada et al. (1998) evaluated the systemic and/or local immunotoxic potential of VCN and demonstrated that VCN induces immunosuppression as evident by a decrease in the plaque-forming cell response to SRBCs, a marked depletion of spleen lymphocyte subsets, as well as bacterial translocation of the normal flora leading to brachial lymph node abscess. These results suggested that VCN has a profound immunosuppressive effect which could be a contributing factor in its gastrointestinal tract carcinogenicity.

Acute exposure to high levels of hydrogen sulfide (H_2S) is life threatening while long-term exposure to ambient levels of H_2S elicits human health effects. In a study by Saeedi et al. (2015), they studied the harmful effects of long-term exposure to low levels of H_2S on human blood cells. In their study, adult workers from Iran who were occupationally exposed from a natural gas processing plants to 0−90 ppb H_2S for 1−30 years were studied. A control group of males who were not in contact with H_2S was also studied. For all participants, hematological profile including total hemoglobin and red blood cell count, sulfhemoglobin and methemoglobin levels were measured. The methemoglobin and sulfhemoglobin levels were significantly higher among workers who were exposed to sulfur compounds than the control group. There was a major difference particularly in sulfhemoglobinemia level among exposed groups. Thus, they have concluded that long-term exposure to even low levels of H_2S in workplaces exhibited both physiological and toxicological roles which may have potential harmful effects on human health.

42.7 Concluding remarks and future directions

CW was on a massive scale during World War I. Chemical weapons are cheap, can cause mass casualties, and are relatively easy to produce, even by developing nations. They were used most recently by Iraq during the Iran−Iraq War, as well as in terrorist attacks. The health effects of chemical weapons on society makes them ideal for terrorism, as shown by the release of nerve gas in the Tokyo subway system by members of the Aum Shinrikyo in 1995. The immune system could be extremely vulnerable to these CWA. As the field of immunotoxicology is developing rapidly, this chapter reviews the impact of CWA on various aspects of the immune system.

The immune response is associated with rapidly multiplying cells and the synthesis of regulatory/effector

molecules and the immune system works as an amplifier for this integrated information network. Immunologic tissue damage can result from activation of the cellular and biochemical systems of the host. The interactions of an antigen with a specific antibody or with effector lymphocytes trigger the sequence of humoral and cellular events to produce the pathophysiologic effects that lead to tissue injury or disease. Stem cells often appear to be sensitive targets for therapeutic and environmental toxicants, most likely because of their rapid proliferation. Certain chemical exposures and doses that do not affect other organs can result in immune dysfunction. Xenobiotics that are toxic to the myelocytes of the bone marrow can cause profound immunosuppression due to loss of stem cells. Humans are now under sustained and increasing pressure from xenobiotic exposure. Xenobiotics can stimulate the immune system as antigens by provoking a substantial immune response. Even mild disturbances of this network could result in detrimental health effects. The influence of the xenobiotics on the immune system could be either suppressive or enhancing. The former leads to immunosuppression with consequent increased susceptibility to infection and cancer. The latter is associated with the development of an autoimmune reactivity such as delayed hypersensitivity, atopy, systemic or organ-specific immunopathology, and granuloma formation. It is likely that the overall immunosuppressive effects of xenobiotics are caused by interference with cellular proliferation and differentiation, downregulation of cytokine signaling, and enhanced apoptosis of immune cells. In contrast, autoimmune reactions are induced by abnormal activation of immune cells followed by dysregulated production of cytokines resulting in harmful inflammatory response (Gulati et al., 2016).

Xenobiotics can act as immunogens to stimulate the production of specific immunoglobulins as a part of an immune response. Specific immunoglobulins might be used as markers of exposure to specific xenobiotics. Attempts must be made to conduct basic research to address the cellular and molecular mechanisms of the immunomodulatory action of various xenobiotics. The newly emerging technologies such as genomics, proteomics, and bioinformatics will certainly be helpful to investigate the interactions between the immune system and xenobiotics in their full complexities. Toxic compounds may be antigenic or act as haptens and can evoke an antibody response. If these antibodies bind to the determinant on the parent molecule which is responsible for causing toxicity, then it can lead to biological inactivation of the parent molecule and thereby prevent toxicity. This may constitute an immunological antidote approach to neutralize the toxicity of certain compounds. Thus, passive administration of the antibodies may be used to prevent the toxic effects of the specific compound, and this approach may be useful in biological or CW to protect against the toxicity of known chemicals or toxins. The antibodies can also be used to protect industrial workers against the toxic effects of known chemicals or gases during accidental exposure. Although this assumption seems logical, it will involve elaborate and time-consuming research to identify the site of the parent molecule responsible for causing toxicity, to chemically link the molecule with a large protein molecule which should be immunogenic but not toxic, and to screen various antibodies raised for their capacity to neutralize or prevent the toxicity of the compound.

As CWAs are a threat to mankind, they have been widely condemned since their first use on a massive scale during World War I. In 1993, the United States signed the Chemical Weapons Treaty, which required the destruction of all chemical weapon agents, dispersal systems, and production facilities by April 2012. The US destroyed 45% of its stockpile of chemical weapons by 2007. As of 2012, stockpiles have been eliminated at seven of the nine chemical weapons depots and 89.75% of the 1997 stockpile had been destroyed by the treaty deadline of April 2012 and the rest was to be destroyed by 2017. The most recent arms control agreement in International Law, the *Convention of the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction*, or the Chemical Weapons Convention, outlaws the production, stockpiling, and use of chemical weapons. It is administered by the Organisation for the Prohibition of Chemical Weapons (OPCW), an intergovernmental organization based in The Hague. However, in early 2007, multiple terrorist bombings had been reported in Iraq using chlorine gas and these attacks wounded or sickened more than 350 people. Thus, in view of the current global scenario, it appears that use of CWAs may continue in different types of warfare as these agents are not only inexpensive but easy to disseminate with the help of unsophisticated devices. Hence the medical profession should assemble on a common platform through globally recognized organizations like the WHO and put in efforts to monitor, research, and study the scientific and medical aspects of CWAs in the interest of humans. There should be updating of guidelines on the prevention and management of CWA-induced insults to reduce morbidity and mortality. Nations worldwide should ensure that adequate supplies of antidotes (wherever available), protective equipment, and decontamination devices are available in adequate quantities and at all times.

The impact of CWAs on the immune system is enormous and CWA exposed individuals are prone to immune system-mediated diseases. Such immunological disorders are growing at epidemic proportions, which requires the development of aggressive and innovative treatment

approaches to cope with such maladies. Developing vaccines, by recognizing the molecular patterns on some xenobiotics, can be a viable approach to tackle the issue and could be the most critical challenge for the research community. In future, more research findings with better data showing the mechanisms of nonimmunologically mediated sensitization, including identification of the cells and mediators involved, is highly recommended. Furthermore, one of the great therapeutic opportunities for the survivors of war is organ transplantation. However, immune system-mediated graft rejection remains the single greatest barrier to widespread use of this approach. In this chapter, the various mechanisms of CWA-induced immunotoxicity are discussed. The effects on different cell types and interference with immune responses, ultimately leading to immunotoxicity as well as sensitizing capacity, are also highlighted. Thus, the need of the hour is a multisectorial approach involving health, defense, agriculture, and environmental specialists, with clearly defined roles of each, for establishing and maintaining effective, robust, and sustainable strategies to counter this threatening situation. Further, physicians and scientists trained in immunotoxicology and environmental health research are needed in the private and academic sectors to help develop expertise in this area. Understanding and perception of risks associated with CWA exposure, especially as related to the immune system, should be fostered through various mechanisms, including the mass media.

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Chapter 43

Health effects of nuclear weapons and releases of radioactive materials

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43.1 Introduction

Humankind and conflict between individuals, that is, warfare, very likely share a common evolution. In the 9th century, the nature of warfare was changed forever when the Chinese invented gun powder. Gun powder could be used to propel projectiles that killed people. Over the centuries a wide range of chemicals were discovered that were increasingly effective in releasing explosive energy. These reactions could be controlled to produce weapons that injured and killed people with projectiles and, yet later, with blast waves. In World War I, the use of explosive weapons was joined by the use of weapons that released chemicals that caused immediate injury and death. Most of the chapters in this textbook are concerned with chemical weapons. This chapter addresses the effect of nuclear weapons and releases of radioactivity. A separate chapter on nuclear weapons and the health effects of blast and ionizing radiation is appropriate in view of their tremendous impact on international politics over the past three-quarters of a century from the end of World War II to the present time.

Warfare, war, and weapons are words that automatically evoke fear and concern. In a similar fashion, the use of the words atomic, nuclear, radiation, and radioactivity, also evoke fear and concern. These words are frequently joined together as in nuclear or atomic weapons or releases of radioactive materials from a nuclear reactor or some other kind of nuclear accident. The fear that these words evoke is grounded in the wide recognition that exposure to ionizing radiation can cause acute injury to living creatures and, with sufficient levels of exposure, cause death in days or weeks. With lower levels of radiation exposure and dose, deaths occur later. At even lower radiation doses, deaths will shorten lifespan with deaths occurring late in life primarily related to an increased incidence of cancers. To provide context, it is important to recognize that cancers are a frequent cause of death, especially late in life, in all mammalian populations. Nonetheless, the word cancer evokes fear and concern.

Concern for the health effects of accidental releases of radioactivity, such as occurred on April 26, 1986, in the reactor accident in Chernobyl, Ukraine and on March 11, 2011, in the Fukushima tsunami incident, led to the evacuation of large populations, thus, adding Chernobyl, Fukushima, and evacuation to the list of words evoking fear. Visual images such as that of a mushroom cloud from a nuclear explosion will also evoke fears.

The level of fear associated with the word radiation is so great that even when radiation is used in a beneficial manner, such as a medical diagnostic procedure or to treat diseases, including treatment of cancer, patients may have concern that potential adverse effects may be greater than the benefits of the radiation-based medical procedure. In some situations, patients may even personally question the advisability of having recommended medical procedures performed that are identified as involving radiation.

The power of the words, atomic, nuclear, radiation, and radioactivity, is such that they are sometimes purposefully used to create fear and concern. I submit that opponents of the use of nuclear reactors to generate electrical power have sometimes used these words to create concern for the use of nuclear reactors. Indeed, in some instances opponents of nuclear-generated electrical power have purposefully equated nuclear reactors with nuclear weapons, although they are vastly different potential sources of radiation exposure. In some circumstances, the intent of some individuals is to arouse fear and concern even in the absence of any radiation exposure or with radiation exposures that are unlikely to produce adverse health effects. It is apparent that fear alone, in the absence

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of physical harm, is a potent weapon. I am sure this fact is well known to terrorists.

In recent years, increased use has been made of the phrase—weapons of mass destruction. This includes nuclear weapons. Indeed, nuclear weapons are widely regarded as the premier weapon system for causing massive destruction and numerous casualties and deaths, as occurred with the bombings of Hiroshima and Nagasaki, Japan, with nuclear bombs. This destructive capability relates to four components of an explosion of a nuclear weapon: (1) blast or overpressure, (2) thermal radiation, (3) direct ionizing radiation, and (4) residual radioactivity that may be transported meteorologically downwind of the site of the nuclear explosion. It is remarkable that concern for nuclear weapons most frequently focuses on the health effects of direct exposure to ionizing radiation from weapons and residual radioactivity. This chapter appropriately notes the substantial health effects of blast and thermal radiation releases in causing massive injuries and mortality.

This chapter starts with some basic concepts and definitions, and then proceeds with a historical review of key events and activities related to the development of our current extensive knowledge of ionizing radiation and its health effects. This will include the period from Wilhelm Conrad Roentgen's discovery in 1895 of X-rays to 1940, an era which was dominated by the increasing use of radiation in medicine. The second era, beginning in 1940, is anchored by the discovery of new elements such as plutonium, and the development and detonation of the first nuclear weapons. Over the last three-quarters of a century, from 1945 to the present, there has been a marked expansion of the use of radiation in medicine, the development and use of nuclear reactors globally to generate electricity, the use of nuclear reactors to power naval vessels, and the development and stockpiling of enhanced nuclear weapons by many nations.

The development of new applications of nuclear radiation in society was accompanied by substantial research on its adverse health effects. This new science was used to inform judgments on standards and regulations to limit occupational and environmental exposures to radiation and radioactivity. Over the past century no potentially hazardous agent has been more extensively studied than ionizing radiation. In my opinion, our level of scientific knowledge of the short- and long-term health effects of exposure to ionizing radiation from external sources or internally deposited radionuclides exceeds our knowledge of the hazardous properties of any other agent or class of agents used by society. Radiation standards and regulations used around the world to limit the exposure of workers and the public are based on a huge body of scientific knowledge.

43.2 Conceptual framework

Hazard and risk are two other words used frequently in this chapter and elsewhere in this textbook. Unfortunately, hazard and risk have frequently been used interchangeably over the past 50 years, especially in the United States, although they have come to have distinctly different definitions. A hazard is any agent or circumstance that has the potential for causing damage, harm, or adverse health effects to someone or something. Hazard is a French word that was probably borrowed from Arabic "az-zahr" meaning "the dice" or "one of the dice." In conventional practice an agent may be classified as hazardous or of causing a specific hazard, such as cancer, without reference to any particular exposure. In contrast, risk is defined as the probability of a particular kind of harm, such as death or occurrence of cancer, from a particular exposure circumstance.

A conceptual background for this chapter is provided in Figs. 43.1–43.3. The risk characterization paradigm

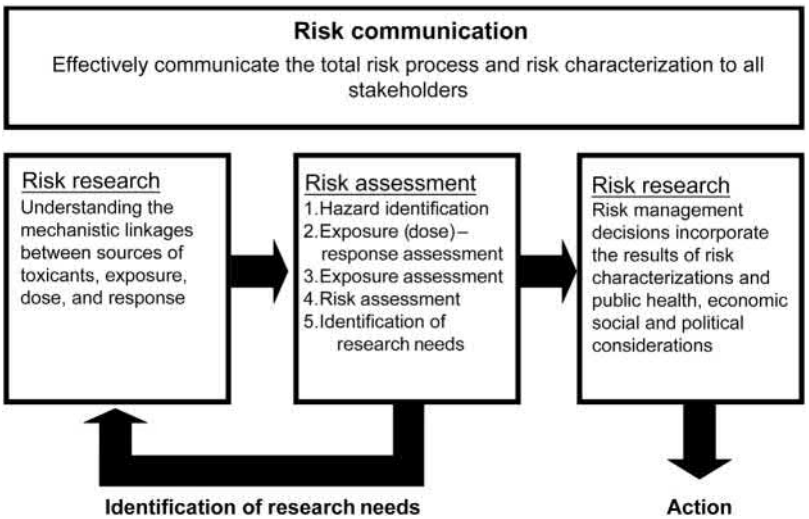


FIGURE 43.1 The risk paradigm that is widely used around the world.

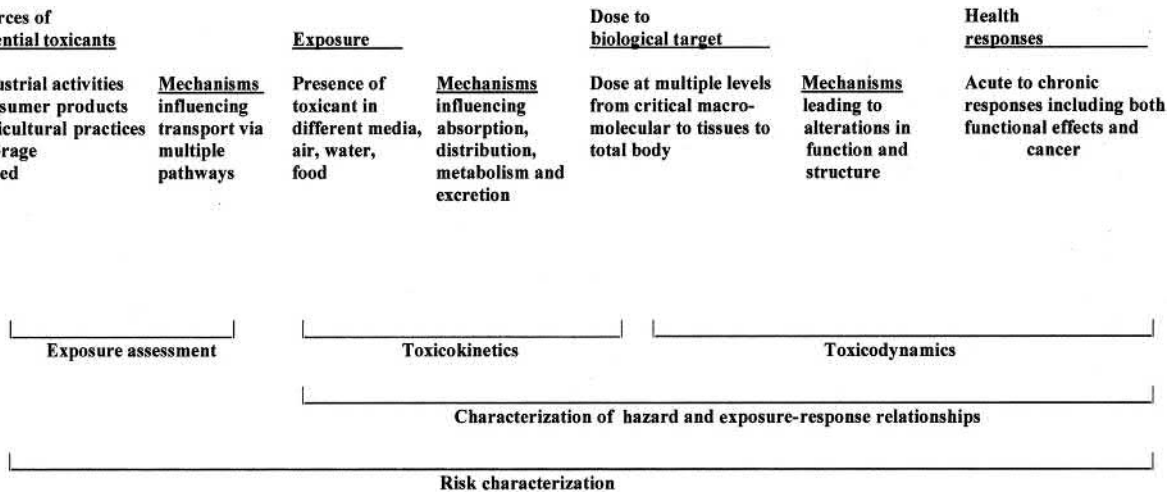


FIGURE 43.2 An expanded risk characterization paradigm.

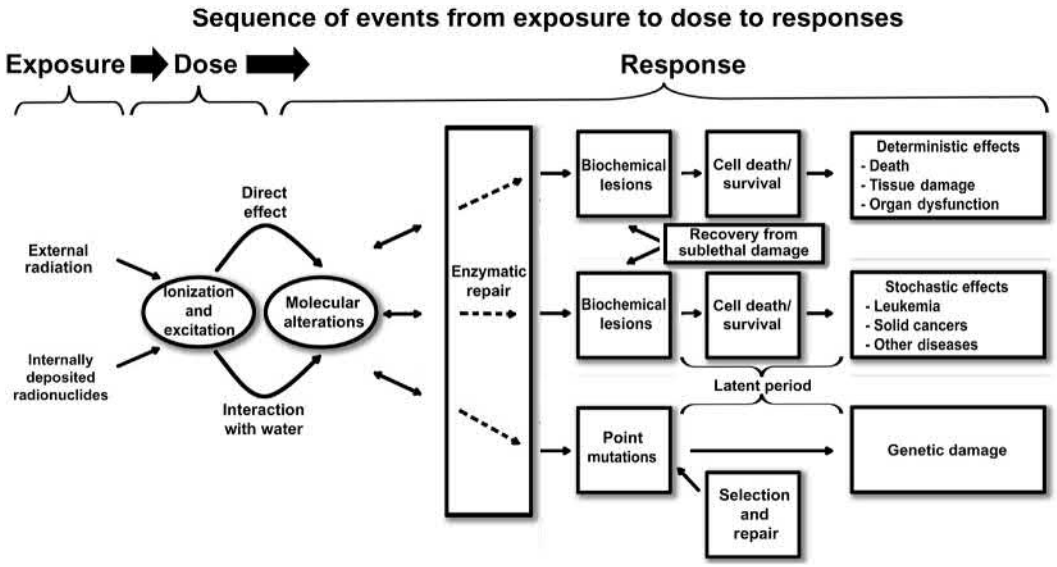


FIGURE 43.3 Conceptual framework linking events from exposure to external sources or internally deposited radioactivity to dose to biological responses. *Adapted from Bushong, S.C., 2017. Radiological Science for Technologists: Physics, Biology and Protection. eleventh ed. 688 pgs, St. Louis, MO: Elsevier/Mosby.*

shown in Fig. 43.1 can be used for all kinds of potentially hazardous agents; it is not unique to radiation. Indeed, I postulate that research on the health effects of ionizing radiation has been a major contributor to the development of the risk paradigm (McClellan, 1999, 2014, 2019).

An expanded risk characterization paradigm is shown in Fig. 43.2, extending from sources of potential toxicants such as ionizing radiation and radioactive materials to health responses. In the world of chemicals, exposure and dose are frequently used interchangeably, which is inappropriate. With ionizing radiation and radioactive materials, the distinction between exposure and dose is well known and clearly illustrated in Fig. 43.2. Additional detail is provided in Fig. 43.3. In recent decades, increased attention has appropriately been given to mechanisms or modes of action of toxicants. This concept has been ingrained in the radiation sciences since soon after the harmful effects of ionizing radiation were discovered. It is often not fully appreciated that mechanistic considerations relate both to the linkage (1) between exposures and dose, that is, toxicokinetics, and (2) between dose and health response, that is, toxicodynamics. I have used toxicokinetics and toxicodynamics deliberately, rather than pharmacokinetics or pharmacodynamics, as I believe these latter terms should be used only for pharmaceuticals.

We all live, play, and work in a world of hazardous agents and circumstances that if not properly controlled can produce health effects over and above those occurring from natural circumstances. Moreover, endogenous events in the absence of external factors such as exposure to radiation or chemicals can cause cancer. To state the obvious, birth is ultimately followed by death and the questions are when death will occur and from what causes. The disease processes and diseases that occur in life are rarely unique to any agent, including radiation.

It is important to recognize that exposure to different agents may cause the same kinds of disease. For diseases occurring soon after exposure to a hazardous agent, the causative nature of the radiation—effect relationship may be circumstantial and quite clear. Identifying a causal linkage between exposure to a hazardous agent and a disease, such as cancer occurring late in life, becomes much more challenging. Indeed, many diseases, such as cancers, are multifactorial in their origin. Thus, a critical issue for many situations is the extent to which there is an excess of disease associated with exposure to a specific agent or circumstance, such as ionizing radiation, relative to a background incidence occurring naturally and unknown etiology.

43.3 Nomenclature

Some key definitions are provided in Table 43.1 for words and phrases regularly used in radiation science and used throughout this chapter.

A concise summary of key units used in radiation science and radiation protection schemes is provided in Table 43.2. The earliest units, shown as old units in the table, are sometimes referred to as conventional units. These have been superseded by the newer International System of Units first developed and used in Europe and more slowly accepted in the United States. In general, when referencing previously published research findings, I will use the units used by the original authors. The reader should be mindful that different prefixes are used,

TABLE 43.1 Key definitions in radiation science and the effects of radiation on health.

Alpha particles	A charged particle consisting of two protons and two neutrons bound together into a particle identical to a helium-4 nucleus
Beta particles	A charged particle of very small mass emitted spontaneously from the nuclei of certain radioactive elements. Physically, a beta particle is identical to an electron moving at high velocity
Blast wave	A rapid change in atmospheric pressure followed by negative pressure. It travels faster than sound
Elements	One of the distinct, basic varieties of matter occurring in nature
Fission	Electromagnetic radiation of variable energy originating in atomic nuclei. Physically, X-rays and gamma-rays are identical.
	The process whereby the nucleus of a particular heavy element following absorption of a neutron splits into two nuclei of lighter elements with release of substantial energy. Controlled and sustained fission occurs in a nuclear reactor. Fission of Pu^{239} and U^{235} is a key component of the detonation of nuclear weapons
Fusion	The process whereby the nuclei of lighter elements, especially those of hydrogen (deuterium and tritium), combine to form the nucleus of heavier elements with the release of substantial energy. A thermonuclear weapon involves fission followed by fusion
Gamma-rays	Electromagnetic radiation of variable energy originating in atomic nuclei. Physically, X-rays and gamma-rays are identical
Isotopes	Forms of the same element having identical chemical properties but differing in atomic mass (due to different numbers of neutrons in their respective nuclei) and nuclear properties (radioactivity)
Nuclide	An atomic species of an element distinguished by the composition of its nucleus, that is, the number of protons and neutrons
Radioactive half-Life	The time required for the activity of a given radionuclide to decrease to half its initial value due to radioactive decay
Radionuclide or radioisotope	An isotope or nuclide that is unstable and will spontaneously decay by emission of alpha or beta particles
Thermal radiation	Steep thermal gradient of energy released in a nuclear explosion
Transmutation	Conversion of one chemical element into another electro-magnetic radiation of variable energy produced by slowing down fast electrons. X-rays can penetrate the body and be absorbed or pass through the body
X-rays	

TABLE 43.2 International units for radiation and radioactivity compared to old units.

International Units	Describes	Old units
Becquerel (Bq)	Radioactivity, the spontaneous decay of atomic nuclei 1 Bq = 1 disintegration/second	Curie = 3.7
		$X10^{10}$ Bq
Gray (Gy)	Dose to tissue 1 Gy = energy uptake of 1 joule/kilogram	Rad = 10 mGy
Sievert (Sv)	Effective dose,[a] dose normalized to effects of gamma radiation by applying a radiation weighting factor (WR) based on the relative biological effectiveness (RBE) of the radiation of interest, to the absorbed dose in an organ or tissue to derive the equivalent dose. The effective dose is usually expressed as millisievert (mSv) 1 mSv = 10^{-3} Sv	Rem[b] = 10 mSv

[a] *Allows conversion of dose from gamma, beta, or alpha radiation to a standard unit.*
[b] *Rem, originally based on Roentgen Equivalent Man.*

such as with Curies or Becquerels, to span many orders of magnitude from micro to milli levels of the base unit. I submit that sometimes authors express the potency of radiation exposure using micro or milli levels of the base unit to exaggerate the apparent potency of the exposure.

43.4 Sources of radiation dose

In considering the potential exposure from any specific incident, it is important to recognize that we all live in a sea of radiation from both natural sources and human activity (Table 43.3). It may be noted that about half of the radiation dose for a member of the US public comes from background and about half from anthropogenic sources. It is important to recognize that radiation exposures are readily measurable. Likewise, the radioactive content of samples is readily measurable. It is important to emphasize that the mere measurement of radiation exposures or the radioactive content of a sample does not directly equate to a health risk.

43.5 Key early events in radiation science

Key early events in the field of radiation science, including those resulting in evidence of radiation-induced health effects, have been well documented. Book chapters I authored provide a succinct review of the subject (McClellan, 2014, 2019). Thus, for this chapter, I will only provide a summary of some of the key events relevant to considering the health effects of nuclear weapons and potential releases of radioactive materials.

Wilhelm Conrad Roentgen, in 1895, discovered a new kind of ray emitted by a gas discharge tube that could blacken photographic film. Because the rays represented the unknown, he called them X-rays. X-rays were soon used diagnostically in medicine and therapeutically to treat cancer. Both diagnostic and therapeutic applications of radiation are an integral part of the practice of modern medicine.

In 1896, Antoine Henri Becquerel discovered radiation emissions from uranium compounds. In 1898, Pierre and Marie Curie isolated polonium and radium from pitchblende ore containing uranium and its decay products. They coined the term radioactivity to describe these materials and to distinguish them from radiation emissions.

The pitchblende ore and uranium from many of these early experiments came from mines in Schneeberg and Joachimstal in the Erz Mountains of Central Europe. As early as the 1400s, it was known that miners in this district frequently developed a chronic respiratory malady. In 1879, the German investigators Härtung and Hesse showed that a majority of the deaths of these miners were due to lung cancer. In the 1940s, this experience would unfortunately be repeated with observation of an excess of lung cancer in uranium miners, especially those who also smoked, working on the western slope of the Rocky Mountains in the United States. One of the benchmark effects of exposure to ionizing radiation was established—alpha particle irradiation of the bronchial epithelium causes an excess of lung cancer.

In the early 1900s, it was discovered that trace quantities of radium and thorium salts could be mixed with phosphors which resulted in phosphorescence. Soon, young women were employed to paint luminous watch and instrument dials. They tipped the fine brushes on their lips and subsequently ingested radium and thorium. Many of these women died from acute injury to their bones and hematopoietic tissues. Others survived this acute injury only to die later from cancer. A recent book, *The Radium Girls*, provides vivid descriptions of working conditions and health outcomes in that time period (Moore, 2016). Thus, another benchmark effect of ionizing radiation was established—alpha particle irradiation of the skeleton and bone marrow causes bone cancer and leukemia.

TABLE 43.3 Relative contribution of different radiation sources to the average radiation dose received by the US public for 2006.

Background	Millisieverts (mSv)	Percent
• Space radiation	0.31	5
• Internal radionuclides	0.31	5
• Terrestrial dose	0.19	3
• Radon and thoron	2.29	37
Background	3.10	
Anthropogenic		
• Computer tomography (CT)	1.49	24
• Nuclear medicine	0.74	12
• Interventional fluoroscopy	0.43	7
• Conventional radiography/fluoroscopy	0.31	5
• Consumer products	0.12	2
• Occupational	<0.1	<0.1
• Industrial	<0.1	<0.1
Anthropogenic	3.10	

Source: Data from National Council on Radiation Protection and Measurements (NCRP) Report 160, Ionizing Radiation Exposure of the Population of the United States (2009).

By the 1920s, it was apparent that exposure to high doses of X-rays or gamma rays could cause acute injury to multiple tissues and with sufficiently high doses with extended periods of observation, an excess of cancer was observed, a third benchmark situation for radiation-induced disease.

On the eve of World War II, Paul S. Henshaw, on the staff of the only recently created US National Cancer Institute, published a seminal paper that summarized the then current knowledge on radiation effects. It identified multiple effects that could be grouped as shown in Fig. 43.4. Most significantly, it postulated that two fundamentally different dose–response relationships were operative for radiation-induced effects (Fig. 43.5).

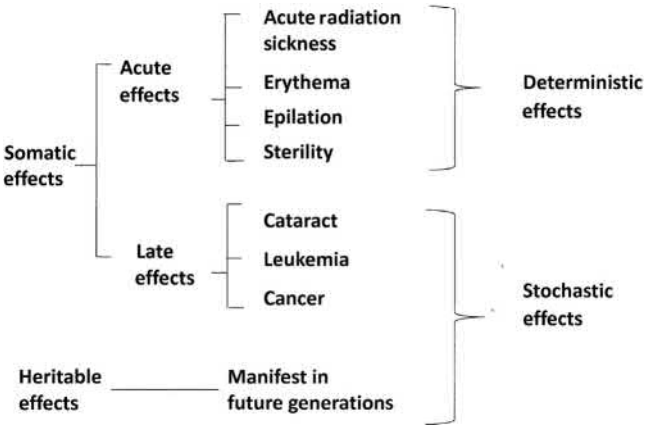


FIGURE 43.4 Major health effects of exposure to ionizing radiation.

43.6 Historical overview of radiation protection standards

Very soon after the discovery of radiation and radioactivity and recognition that radiation could cause harmful effects, action was taken to develop guidance to limit adverse health effects in people using radiation and radioactivity. I have discussed these activities in some detail in a book chapter (McClellan, 2014). To provide context for this chapter, I will briefly review those activities here.

The first radiation protection activities were voluntary and involved defining levels of exposure to X-rays that could or would not harm the skin; in short, the identification of a threshold dose for producing erythema. In 1902, Rollins proposed a specific tolerance dose—"if a photographic plate is not fogged in 7 min the radiation is not of harmful intensity." This represented the first association of an exposure or dose metric with a health outcome. It also represented a joining of the physical sciences with medical science and practice. This was an era in which

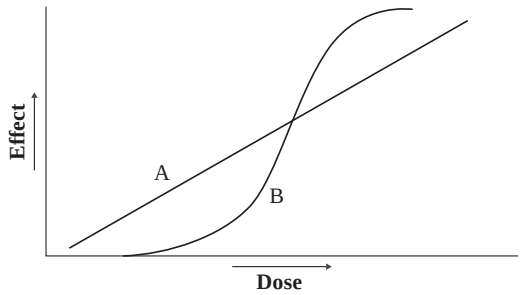


FIGURE 43.5 Schematic rendering of two fundamentally different dose–response relationships for radiation. A—a linear nonthreshold relationship (stochastic) and B—threshold (deterministic) relationship. *Adapted from Henshaw, P.S., 1941. Biologic significance of the tolerance dose in x-ray and radium protection. J. Natl. Cancer Inst. 1, 789–805.*

the disciplines of medical physics and radiological physics were developing with individuals in these disciplines applying their expertise to medical issues. The British took a lead role in developing radiation protection guidance, and in 1925 radiologists and medical physicists convened at the First International Congress of Radiology in London. A second congress followed in 1928 in Stockholm. The radiation protection programs that originated at those meetings evolved into what is now known as the International Commission on Radiological Protection (ICRP) that continues today as a self-governing entity.

An employee of the US National Bureau of Standards, Lauriston Taylor, attended the 1928 Stockholm Congress. He brought back to the United States the radiation protection criteria that had been agreed to at the Stockholm Congress. Taylor soon organized a US Advisory Committee on X-ray and Radium Protection under the auspices of the US Bureau of Standards. This would evolve into the National Council on Radiation Protection and Measurements (NCRP), an independent nonprofit entity that operates under a US Congressional Charter given in 1964. It is governed by a self-elected Council of 60 individuals. The author served on the NCRP Council from 1971 to 2001 and is now a Distinguished Emeritus Member.

The ICRP and the NCRP have both issued numerous reports on radiation protection matters. These reports, in turn, have provided guidance to government agencies, both international and national, for the development of government-authorized standards, regulations, and guidance. It is also appropriate to note three other organizations that have influenced radiation protection guidance. One of these is the United Nations Scientific Committee on Effects of Atomic Radiation (UNSCEAR) that periodically issues reports on various aspects of the health effects of radiation. A second is the International Atomic Energy Agency (IAEA) that operates at the interface of science and policy. The third entity is the US National Academies of Science, Engineering and Medicine (NASEM) subunit, the National Research Council (NRC), that has sponsored various committees such as the Committee on Biological Effects of Ionizing Radiation (BIER). The BEIR reports have had a substantial influence on US government policy and standards dealing with radiation matters.

43.7 Discovery of fission changed the world

Let me turn to the events that triggered a second era in the radiation sciences. In December 1938, German chemists Otto Hahn and Fritz Strassmann sent a manuscript to Natnwissenschaften (Hahn and Strassmann, 1939) reporting they had detected the much lighter element, barium, after bombarding uranium with neutrons. They shared these results by mail with Lisa Meitner, who had lost her German citizenship because of her Jewish lineage and moved to Sweden. Her nephew, Otto Frisch, was visiting with her over the holidays at the end of 1938. Meitner and Frisch (1939) correctly interpreted the findings as nuclear fission, a splitting of the atom. Frisch, who had shifted his interests to biology, proposed calling the process fission as an analogy to binary fission in biological cells and the resulting fission products were called daughter products. The discovery of fission was key to the subsequent development of atomic or nuclear bombs that first used fission and later fission followed by fusion. Nuclear fission was soon verified by an American scientist, Phillip H. Abelson (1939), working at the University of California-Berkeley.

In December 1940, Glenn Seaborg and his colleagues used the cyclotron invented and built by Ernest Lawrence at the University of California-Berkeley, to produce a new element, plutonium, and soon thereafter nine other new elements. It was quickly determined that ^{239}Pu could fission and, thus, it was postulated it could be used to fuel a nuclear bomb. It was already recognized that naturally occurring ^{235}U could be used to fuel an atomic bomb.

In the buildup to World War II, the United States created the Office of Scientific Research and Development to coordinate research aligned with national defense. One of the projects it sponsored was Enrico Fermi's research to demonstrate that a self-sustaining chain reaction of nuclear fission in natural uranium could be produced and the uranium transmutated to higher atomic number elements such as Pu. On December 2, 1942, Fermi and his team, working at the University of Chicago, demonstrated that a sustained chain reaction yielding neutrons could be produced in a uranium pile with carbon black moderation with the reaction stopped by introducing a neutron-absorbing material such as boron.

43.8 The Manhattan Project

In early 1943, the responsibilities of the Office of Scientific Research and Development were transferred to the US Army Corp of Engineers with a new secret entity created, the Manhattan Project. It was headed by Major Andrew Groves, who would soon be promoted to General. A review of the details of the Manhattan Project is beyond the scope of this chapter. Suffice it to note that (1) a new laboratory was created at Los Alamos, NM, to design and build nuclear bombs, (2) new facilities were constructed at Hanford, WA, including water-cooled graphite-moderated reactors to produce ^{239}Pu from uranium and specialized separation facilities to separate ^{239}Pu from the residual uranium and fission products, and (3) new facilities were constructed at Oak Ridge, TN, to produce ^{235}U. Basic research and development activities continued at the University of California-Berkeley and University of Chicago and other institutions were engaged to meet specific needs.

Safety was of paramount concern at all the sites and was heightened by the construction and use of facilities never built before, such as nuclear reactors, specialized chemical separations facilities, and the handling of newly discovered elements such as Pu. Glenn Seaborg told me that when he isolated weighable quantities of Pu he was aware of the experience of the radium dial painters developing bone cancer. He shipped an aliquot of the ^{239}Pu to his colleague, Joe Hamilton, at the University of California-Berkeley, to investigate the toxicity of the new material. A new field of science was initiated.

43.9 The tolerance dose

Each of the Manhattan Project sites faced unique radiation protection issues. Hence, it is not surprising that each of the sites developed specific guidance to protect its workers and the public. The document for the Hanford site was developed beginning in the fall of 1943 by Seymour Cantril, a physician oncologist, knowledgeable of using radiation in cancer therapy, and Herbert M. Parker, a British-trained medical physicist experienced in the use of radiation in cancer therapy. In developing the document, Cantril and Parker drew heavily on the guidance of the ICRP and the US Advisory Committee on X-ray and Radiation Protection, which would later become the NCRP. They also made use of the Henshaw (1941) paper, including the phrase "the tolerance dose," which he used in the title of his paper. Their goal was to release the document before start-up of the first Hanford reactor and the first processing of irradiated fuel. However, publication of the document entitled "The Tolerance Dose" was delayed due to security concerns; it was published on January 5, 1944 (Cantril and Parker, 1945). Key excerpts from the document are quoted below.

"In reviewing the subject of tolerance dose, it is most striking that animal experimental evidence has played practically no part in arriving at present day levels. In summary, there are only three tolerance levels, which have been established and accepted as a working basis for occupational exposure.

0.1 r per day for external X and gamma radiation
-1×10^{-14} curie/cc for radon in the air of working rooms
0.1 μg of radium as the maximum allowable amount deposited in the body of a radium dial painter

Each of these levels has been established by adding a safety factor to the amount, which has been known to produce lasting injury to persons so exposed. It is of interest also to note that in each case the factor does not exceed 10, and is more likely considerably less than 10. Human misfortune rather than animal experimentation were the basis for these levels."

The emphasis on the use of human data, when they are available, as noted in 1945, continues to the present time. Data from experiments with laboratory animals designed and conducted in a purposeful manner have a supporting role. The same may be noted for observations from the use of in vitro systems.

43.10 The first nuclear weapons

On September 26, 1944, the 100-B reactor at Hanford went critical under the watchful eye of Enrico Fermi and was soon producing grams and then kilograms of Pu-239. The 100-B reactor was a 36-foot cube of carbon with transverse channels to hold the uranium fuel slugs and cool the reactor with purified cold Columbia River water. In December 1944, green irradiated nuclear fuel rods were processed at Hanford using the methods developed by Glenn Seaborg and colleagues at the University of Chicago. This was accomplished in large canyon-like buildings about 800 ft in length. Workers called them the "Queen Marys." The first shipment of Pu-239 for use in a bomb was sent from Hanford to Los Alamos in early 1945. Soon the Oak Ridge facility was shipping kilogram quantities of U-235 to Los Alamos.

The bomb design and fabrication activities at Los Alamos came to a crescendo in the summer of 1945. On July 16, 1945, at the Trinity Site in central New Mexico, the first atomic bomb with Pu-239 as the fissile material was tested on a tower under the watchful eye of Robert Oppenheimer, who came from the University of California at Berkeley, to head the bomb design and fabrication team at Los Alamos. On viewing the nuclear explosion, Oppenheimer was reported to have quoted a passage from Bhagavad Gita: "If the radiance of a thousand suns were to burst at once into the sky, that would

be like the splendor of the Mighty One.... I am become death, the shatter of worlds." The world would never be the same!

It is noteworthy that downwind of the Trinity detonation, some cattle, sheep, and burros were grazing. Within weeks, the backs of some of the dark-skinned Hereford cattle and burros were showing white spots, a result of beta burns from radioactive fallout particles. Soon the US government bought some of the cattle and burros and shipped them to Los Alamos and Oak Ridge for observation. This is probably the first government indemnification program related to nuclear activities. It is not known if any observations of whole-body radioactivity were made on the Los Alamos officials who likely enjoyed steaks from the slaughtered cows. It would be a few years before the first whole-body counter to measure body burdens of radioactivity would be developed. It is known that the burros enjoyed long lives in Tennessee and when necropsied decades later by the late Clarence Lushbaugh were found to be free of cancer, an unexpected finding.

On August 6, 1945, a previously untested U-235 fueled bomb, called "Little Boy" was detonated over Hiroshima, Japan. On August 9, 1945, a Pu-239 fueled bomb, called "Fat Man," similar to the device tested at the Trinity Site, was detonated over Nagasaki, Japan. Within a very short period of time Japan surrendered, ending World War II.

It is noteworthy that shortly after the atomic bombs were dropped on the Japanese cities the workers at Hanford were informed of the role of Hanford and workers were thanked for their special efforts with letters from the US Secretary of War and the President of DuPont, the prime contractor at Hanford. Special note was made of the unique facilities and safe work practices. As an aside, DuPont had been selected as the prime contractor at Hanford based on its unique experience designing, constructing, and operating facilities producing chemical explosives.

43.11 Post-World War II nuclear weapons development and testing

After World War II, the United States continued to develop, manufacture, and test nuclear weapons. On July 1 and 25, 1946, the US detonated nuclear fission bombs in "Operation Crossroads" conducted at Bikini Atoll in the Pacific.

On January 1, 1947, the activities of the Manhattan Project were shifted to a newly created civilian agency, the US Atomic Energy Commission. Ostensibly, this was a shift from military to civilian control of nuclear weapons and nuclear energy. The history of the AEC has been reviewed in depth by Hewlett and Anderson (1962) and Hewlett and Duncan (1969). In 1974, the AEC activities related to commercial reactor development were transferred to a newly created entity, the US Nuclear Regulatory Commission. Other AEC activities were continued under the Energy Research and Development Administration (ERDA). In 1977, ERDA became the US Department of Energy.

As World War II came to a close, the Union of Soviet Socialist Republics (USSR) accelerated its effects to develop atomic bombs. This included development of ^{239}Pu-producing facilities at Mayak, Russia, that were very similar to those developed at Hanford. On August 29, 1949, the USSR detonated a "Fat Man" type, ^{239}Pu-fueled bomb. The nuclear arms race was on and accelerating!

Soon other nations joined the race, which continues to the present time with large stockpiles created of both fission and fusion bombs with potential for delivery by aircraft, naval vessels from silos, or from ground-based mobile units. These are frequently referred to as the nuclear triad. Nuclear testing activities by the United States continued in the South Pacific. Later tests were also conducted at a special test site in Nevada. There were both surface and underground detonations. By early 1962, the US had conducted about 1000 tests of nuclear weapons. The United Kingdom conducted nuclear tests in Australia, the French in North Africa and the Pacific, the USSR in north central Asia, and later, India would conduct underground nuclear tests on the Indian subcontinent, China would detonate multiple devices in northern China, and North Korea would conduct underground tests.

After a brief tacit moratorium on testing in 1958–61, the USSR resumed testing. The United States followed suit with a series of 31 tests (Operation Dominic) in 1962 in the South Pacific. Those 31 tests had a total yield of 38.1 megatons. On May 6, 1962, a nuclear-powered submarine, the USS Ethan Allen, as a part of Operation Dominic in a test code named Frigate Bird, fired a nuclear warhead-armed Polaris ballistic missile from its location south of Hawaii toward Johnston Island in the South Pacific. The reentry vehicle traveled 1200 miles in 12.5 min. The fission/fusion weapon, 18 in. in diameter, 46.6 in. long, and weighing 717 pounds was detonated in the air at 11,000 ft, releasing about 600 kt of energy. For comparison, the atomic bomb dropped on Hiroshima was estimated to have released 16 kt of energy. I encourage readers to view on the internet videos of the detonation taken from a submarine located about 30 km from the detonation. The explosion was awesome! It should be noted that many nuclear devices and missile delivery systems have been tested independently. Frigate Bird is the only test of an integrated system from launch to detonation. A crew member on the Ethan Allen related: "the detonation was right on target in the old pickle barrel just as planned."

The Frigate Bird weapon had a yield to weight ratio of 1.84 kt/kg. It has been reported that some nuclear weapons have yield to weight ratios on the order of 5.0 kt/kg. It is apparent that weapons with enormous destructive power can be delivered to targets anywhere in the world. It is difficult for me to envision a "small nuclear weapon" that will not have a devastating impact on a substantial area around a target.

The use of nuclear systems extended into space in the 1960s. The US initiated a program to develop Space Nuclear Auxiliary Power (SNAP) systems. This included the use of nuclear reactors and radionuclides to generate electrical power. ^{238}Pu was quickly identified as the radionuclide thermal source of choice. The configuration of the ^{238}Pu fuel was changed after one SNAP device (9A) failed to achieve orbit and burned up over Africa. These radionuclide- fueled SNAP units are still being used. The Soviet effort using nuclear reactors to generate electrical power was apparently discontinued after one failed and burned up over Canada.

43.12 Contemporary nuclear activities

In mid-2019, it became apparent that Russia was developing and testing a nuclear-powered missile that could carry a nuclear weapon. The program became public when an accident releasing radioactivity occurred. The US had conducted a similar program centered at Los Alamos in the 1960s and 1970s. However, that program was discontinued before a nuclear-propelled rocket was fully developed and tested.

Public media reports indicate that Iran is continuing to pursue the development of ^{235}U-fueled nuclear weapons and North Korea has continued to develop and test ^{239}Pu-fueled nuclear weapons. What was once called a nuclear arms race is now best described as an on-going nuclear weapons political brawl!

In addition to the existence of stockpiles of nuclear weapons containing ^{239}Pu and ^{235}U, substantial quantities of fission products and uranium from reprocessing of spent fuel from nuclear reactors exists. In addition, in the United States there are substantial amounts of spent irradiated fuel assemblies that have not been processed and are in interim storage. This situation exists because in the administration of President Jimmy Carter a decision was made that the United States would discontinue the reprocessing of spent nuclear fuel.

It is also noteworthy that a number of nuclear-powered naval vessels have been built over the decades. The concept of using a nuclear reactor to propel submarines was first advanced by Phillip Abelson (Abelson and Abelson, 2007) and soon embraced by Admiral Rickover. Some have also been deactivated. In accord with international agreements, the decommissioned nuclear reactors containing spent nuclear fuel are stored above ground so they are readily visible for observation from space for accountability purposes. A discerning eye can observe many of these units above ground at Hanford in what I call "Admiral Rickover's Graveyard" in homage to the individual who provided leadership for decades for the US Nuclear Navy. Earlier I made reference to the nuclear-powered submarine, the USS Ethan Allen. It was launched on November 22, 1960, and commissioned on August 8, 1961. In the 1980s, in accord with international agreements, its Polaris missile-launching capabilities were deactivated. It was decommissioned at Bremerton, WA, on March 31, 1983, and recycling was completed on July 30, 1999. A part of its hull containing reactor components is now visible at Hanford, WA.

43.13 Blast and thermal effects of nuclear weapons

As I have already noted, the detonation of a nuclear weapon results in release of extraordinary amounts of energy in four forms: (1) thermal energy, (2) blast wave, (3) direct emissions of ionizing radiation as gamma rays and neutrons, and (4) radioactive elements that can be transported downwind of the explosion. It is very fortunate that only two nuclear weapons have been used in war, that is, the bombings of Hiroshima and Nagasaki, Japan, which resulted in thousands of deaths. As will be discussed later, some of the populations of those cities who survived the bombing have been studied extensively. These studies are on-going with support from the governments of Japan and the United States. A description of those activities, *Song Among the Ruins*, was authored by one of the major scientific participants, W.J. Schull (1990).

Since the bombings of Hiroshima and Nagasaki, numerous nuclear weapons have been exploded for test purposes. Several of the nuclear weapons tests resulted in inadvertent exposure of very small populations that have been studied (Conrad et al., 1980). The vast majority of nuclear weapons tested yielded large amounts of physical measurements on the destructive effectiveness of the weapons—it is huge! Radioactive materials formed in the nuclear explosions have been injected into the atmosphere, transported around the world, and studied extensively. A discussion of the results of that research is beyond the scope of this chapter.

This section focuses on the effects of the blast waves produced by nuclear explosions and the direct thermal radiation emissions. Few scientists today are knowledgeable of these effects, even scientists who have spent a career in the radiation field. Ignoring these devastating effects will not make them go away; they are a reality for nuclear weapons!

As one might surmise, a substantial amount of the literature on the effects of nuclear weapons is classified and, thus, not available to the public. However, a nonclassified document, *The Effects of Nuclear Weapons*, was compiled and edited by Samuel Glasstone and Philip J. Dolan (1977). Although the document was published over four decades ago, I have been informed by experts on this specialized subject that the basic concepts presented in 1977 are still highly relevant. It is appropriate to note that the first edition of the nuclear weapons effects document was published in 1950 when the explosive yields of the fission bombs available at that time were equivalent to some thousands of tons (i.e., kilotons) of TNT. By 1957, when a second edition was released, thermonuclear (fusion) weapons had been developed and tested with energy yields in the range of millions of tons (i.e., megatons) of TNT. To provide perspective, a kiloton explosion releases 10^{12} calories of explosive energy. This is equivalent to 3.97×10^9 British Thermal Units of energy.

For the purposes of this chapter it is sufficient to note that the energy yield of every nuclear weapon is specific to that weapon. Moreover, the distribution of any population relative to the nuclear detonation will determine the relative impacts of thermal, blast, direct radiation, and radionuclide releases on exposed populations.

The 1977 Edition of *The Effects of Nuclear Weapons* (Glasstone and Dolan, 1977) contained as a supplement a circular slide rule—"Nuclear Bomb Effects Computer"—that was developed by the Lovelace Biomedical and Environmental Research Institute personnel based on data in the document. The "slide rule computer" can be used to calculate a number of parameters for different nuclear weapons yields; thermal radiation, blast parameters, reflected overpressures, translational velocities for man, initial radiation exposures, and early fallout dose-rates. I strongly suspect that this rudimentary computer has been updated and improved by many governments around the world using contemporary computer technology.

The values of the various physical yield parameters and their related health impacts that are calculated for even low-yield nuclear weapons are staggering! Suffice it to note, all of the individuals near the epicenter of a nuclear weapons explosion, even of low yield, will be killed by thermal radiation, blast pressure, direct radiation, or a combination of these components. A critical issue becomes at what distance from the epicenter will there be any survivors with injuries amendable to treatment, using modern medical procedures. In evaluating the potential effectiveness of such treatment it is important to recognize that the number of immediate casualties could number in the tens of thousands dependent upon the target. The number of survivors with life-threatening injuries could number in the tens of thousands and higher. Thus, it is unlikely that the heroic medical procedures used in modern medical centers to effectively treat a few patients with thermal burns or blast injury will be available to treat large numbers of patients with blast or thermal radiation injuries.

It is important to recognize that blast injuries are not unique to nuclear weapons, explosive devices of all kinds produce blast waves that kill people. This includes improvised explosive devices (IEDs) that have accounted for about 75% of all US military casualties in Iraq and Afghanistan (Belmont et al., 2010). A recent Institute of Medicine Report (IOM) entitled "Gulf War and Health, Vol. 9, Long-Term Effects of Blast Exposures" (National Academies Press, 2014) provides an excellent review of blast effects. The contents of the 2014 report builds on material on the blast effects of blast waves reviewed in an earlier IOM report, "Gulf War and Health, Vol. 7, Long Term Consequences of Traumatic Brain Injury" (National Academies Press, 2009). These reports focused on the effects of relatively small IEDs with explosive yields of a few pounds of TNT to 500 pounds of explosives in the trunk of a sedan to 10,000 pounds in a small box van to 60,000 pounds in a semitrailer. The lethal air blast range from a 500-pound IED is estimated to extend to 100 ft and increases to 600 ft for a 60,000 lbs IED. The reports cover a range of effects from acute deaths to delayed effects observed in survivors.

I strongly encourage individuals interested in blast effects to read and study the 1977 report of Glasstone and Dolan as well as the IOM reports. However, I want to emphasize that the focus in the IOM reports on blast effects from relatively small IEDs and the limited number of individuals in harm's way need to be kept in mind in extending these results to even low-yield nuclear weapons. The destructive potential of nuclear weapons is substantial!

The IOM reports selectively review the experimental data developed under experimental conditions in studies with many mammalian species. Much of this early data was developed under the direction of Clayton S. White of the Lovelace Medical Center in Albuquerque, NM (Bowen et al., 1968a,b; White et al., 1970; Martinez and Stuhmiller, 1999). Those studies conducted at the Nevada test site and using blast tubes demonstrated that the primary blast effects were observed in the thorax and abdomen. Clemedson and Criborn (1955), in a classic post-World War II paper, postulated that these effects resulted from the shock waves' kinetic energy.

A key finding of the Lovelace research was the observation of substantial differences in blast tolerance (24-h survival) among the 12 species studied (Bowen et al., 1968a; Richmond et al., 1967, 1968) (Fig. 43.6). Body size-dependent differences in blast tolerance have been explained on the basis of lung density. The lung density of larger species (humans, monkeys, cats, and dogs) is only about one-half that of smaller species such as

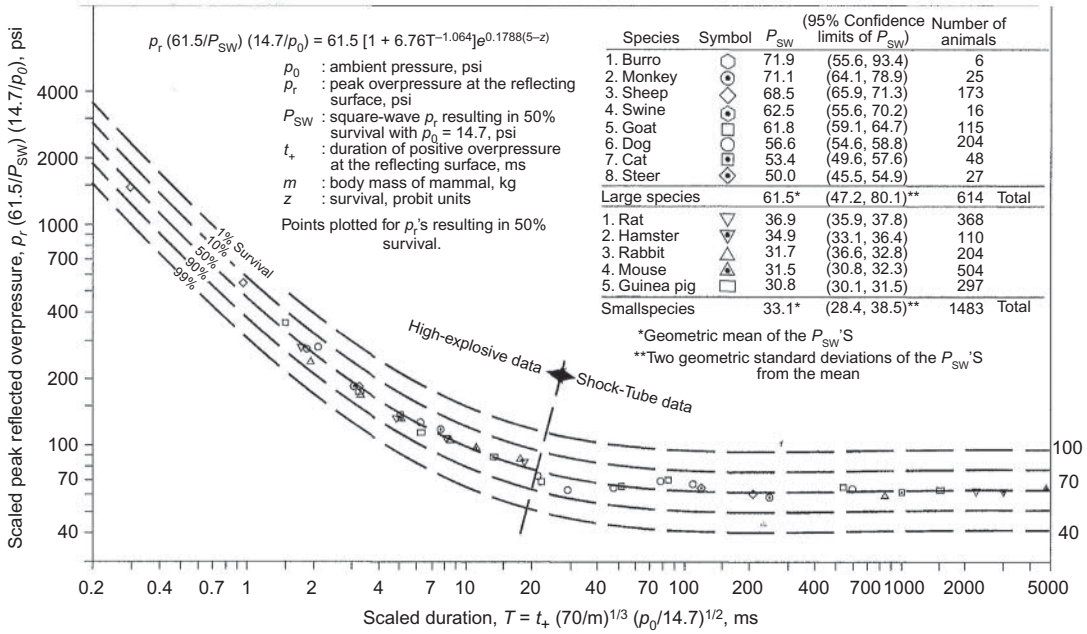


FIGURE 43.6 Survival curves (24-h) applicable to sharp-rising blast waves typical of a nuclear explosion, derived from analysis of data from 12 mammalian species (excluding guinea pigs) (Bowen et al., 1968b).

mice, rats, guinea pigs, hamsters, and rabbits. In contrast, the lung volumes relative to body mass in smaller species were three times greater than in the large species (White et al., 1965).

Despite these documented species differences, many recent studies of blast injury have used mice and sophisticated molecular and cellular approaches. The findings are interesting. However, I question the relevance of some of these findings to the human situation. I urge future investigators to take the time to review literature on blast effects developed over half a century ago using larger species before initiating new research on blast effects using rodent species. The appropriate animal model is as important as using sophisticated methodology. In my opinion, the study of blast effects is a situation where in vitro techniques cannot substitute for whole-animal studies.

43.14 Exposures to radioactive materials and radiation dose

The complex nature of the pathways of exposure for ionizing radiation and radioactive materials is illustrated in Fig. 43.7. To avoid giving undue emphasis to the direct effect of ionizing radiation from explosion of a nuclear weapon, that type of exposure is not illustrated in Fig. 43.7. As will be discussed later, the explosion of a nuclear weapon directly releases fission fragments, beta particles, gamma-rays, and neutrons that can expose individuals. The exposure and associated delivered radiation dose will depend on the individual's distance from the

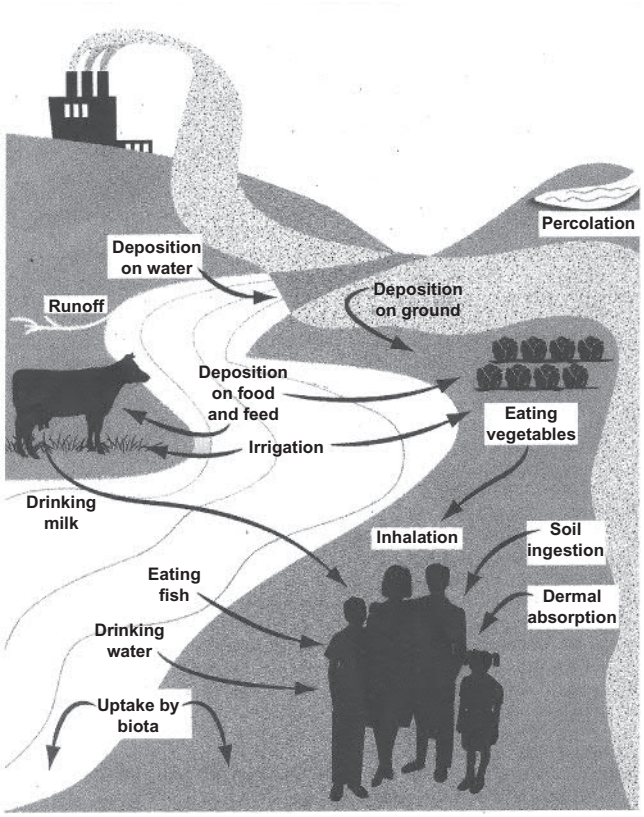


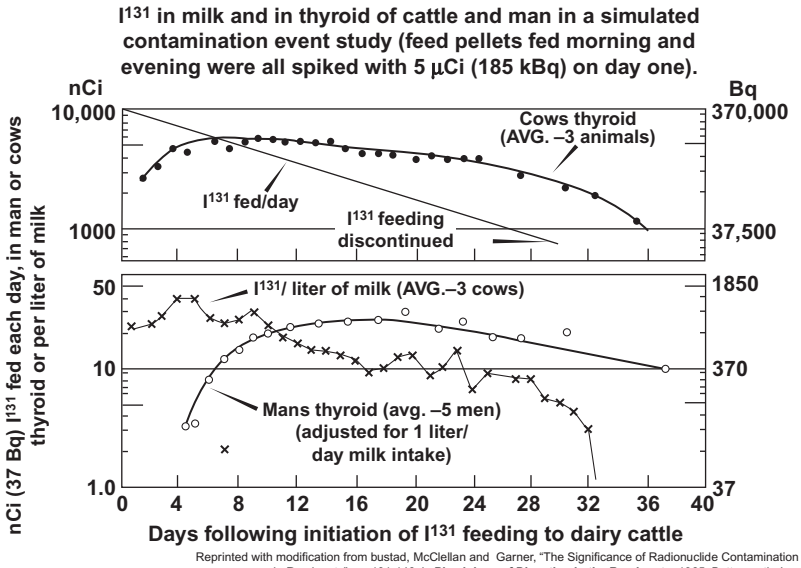
FIGURE 43.7 Schematic representation of pathways for radioativity to reach humans. *Adapted from Paustenbach (2001).*

nuclear explosion and other factors, most notably shielding. Airborne radioactivity, that is, fission products, from a nuclear explosion, a nuclear reactor accident, or other releases of radioactive materials may serve as a source of external exposure. However, intake of radioactivity typically results in the most substantial radiation dose to populations downwind of the nuclear explosion.

One of the most important pathways for exposure of human populations to radionuclides from a variety of accidental releases involves intake of contaminated pasture, grass, or hay by dairy cows, resulting in contaminated milk and ingestion of that milk by humans. As will be discussed later, recognition of the role of this pathway and actions to block it are critically important in limiting population exposures for many kinds of releases of radioactive material. An enormous array of different radionuclides is constantly being produced in a nuclear reactor by fission of the uranium or plutonium fuel or to a lesser extent by neutron activation. A similar array is produced instantaneously in a nuclear fission or fission–fusion weapon. Most of these radionuclides and their radioactive decay products have very short physical half-lives, measured in seconds or minutes, that limit human exposures.

Three radionuclides released in the fission of uranium and ^{239}Pu have decay characteristics (half-lives and emissions) and biological characteristics that are of special concern. Radioiodine, ^{131}I, decays with a physical half-life of 8.05 days and, like stable iodine, is readily adsorbed when ingested and concentrates in the thyroid. Radiostrontium, principally ^{90}Sr with a physical half-life of 28.0 years, behaves like its alkaline earth analogy, calcium, and is readily absorbed when ingested in a soluble form and concentrates in the skeleton. It decays to short-lived ^{90}Y with beta emissions. The beta emissions of ^{90}Sr and ^{90}Y irradiate the skeleton and bone marrow. The alkali metal, ^{137}Cs, behaves like its analog, potassium, and when ingested or inhaled rapidly distributes throughout the body with gamma and beta emissions serving as a source of whole-body irradiation.

Of these three radionuclides, ^{131}I is most important for down-wind exposure of populations to radioactive fallout from detonation of a nuclear weapon, releases from a nuclear reactor accident, or reprocessing of "green" irradiated fuel. Fig. 43.8 relates key data from a study in which ^{131}I was fed to dairy cows to simulate what might occur following a nuclear detonation, a reactor accident, or a nuclear fuel reprocessing release. The amount of radioactivity intake each day is reduced as a result of the 8.05 day physical half- life of ^{131}I. As may be noted, there is a buildup of ^{131}I in the thyroid of the cows and continuous secretion of ^{131}I in the cow's milk. In this study, volunteers drank aliquots of the contaminated milk and buildup of ^{131}I in the thyroid of the volunteers occurred. As an aside, the radiation dose to the thyroid of the volunteers was less than that associated with a chest radiograph given in the early 1960s. The data contained in the figure provides a sound scientific basis for avoiding the processing and consumption of milk from dairy cows after any release of radioactivity until assurance can be given that the levels observed will not result in ^{131}I intakes that exceed dose limits. This kind of prudent action was carried out quite effectively following the Fukushima incident. Unfortunately, this practice was not uniformly followed in areas downwind of the Chernobyl reactor accident. Indeed, people in many areas downwind of Chernobyl were not informed for some time of the occurrence of an accident releasing substantial amounts of radioactivity, including ^{131}I.



Reprinted with modification from bustad, McClellan and Garner, "The Significance of Radionuclide Contamination in Ruminants", pp 131-146, in **Physiology of Digestion in the Ruminants**, 1965, Butterworth, Inc.

FIGURE 43.8 Interrelationships for radioiodine (I^{131}): Intake by cows, concentration in cow's thyroid and milk, and human thyroid. *Adapted from Bustad, L.K., McClellan, R.O., Garner, R.J., 1965. The significance of radionuclide contamination in ruminants. In: Physiology of Digestion in the Ruminant, pp. 131–143. Washington, DC: Butterworth (Bustad et al., 1965).*

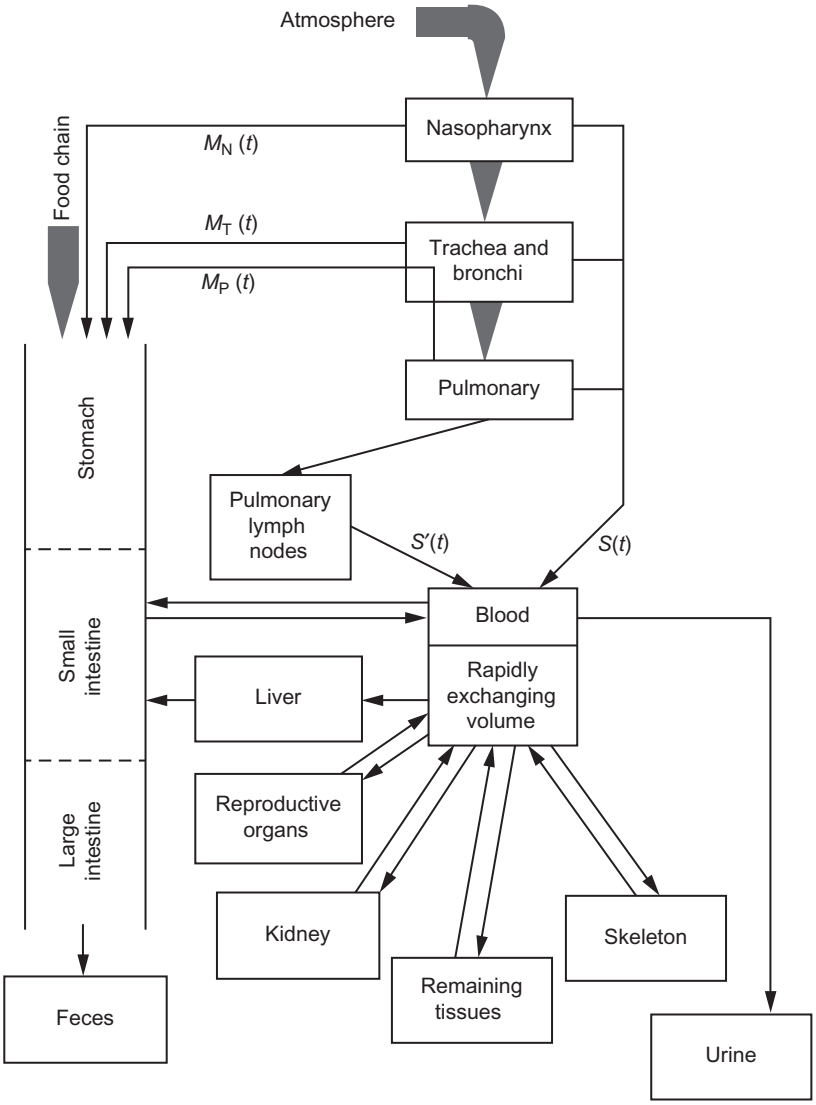


FIGURE 43.9 General model used to simulate organ distribution and retention patterns for inhalation, ingestion, or injection of radioactive elements. Clearance of material from the respiratory tract is considered to involve competing mechanical clearance pathways with material reaching the gastroingestinal tract, $M(t)$ or lymph nodes and dissolution with absorption into blood, $S(t)$. Both rates may be time-varying functions dependent upon the physical-chemical characerristics of the inhaled material. Other transfer rates are considered to be first-order unless better information is available for specific radionuclides and chemical forms (Cuddihy et al., 1976).

As an aside, if radionuclides are inhaled the retention kinetics will be determined by the vector with which the radionuclide is associated. For example, if the aerosol is a soluble chloride, such as $SrCl_2$, the ^{90}Sr will readily reach the bloodstream and be transported to the skeleton. In contrast, if the vector is relatively insoluble fused aluminosilicate particles (FAPs), the ^{90}Sr-FAP will be retained for an extended time in the pulmonary region and irradiate the lung. A general model used for describing the fate and radiation dose for internally deposited radionuclides is shown in Fig. 43.9.

43.15 Radiation-induced health effects

43.15.1 Sources of information on radiation effects

There are multiple sources of information on the health effects of exposure to radiation. As Cantril and Parker (1945) noted at the time they wrote their now classic document, *The Tolerance Dose*, studies of "human misfortune" are a major source of information on radiation effects. In retrospect, each of those pieces of human information with documented adverse effects represents a failure to adequately control past activities and limit radiation exposures. Post World War II, substantial effort were expended conducting epidemiological information on exposed human populations. Key human populations studied following external exposures to X- or gamma radiation are shown in Table 43.4. Key human populations studied following intake of radioactivity are shown in Table 43.5. The quality of those studies was heavily influenced by the quality of dosimetry, epidemiological methodology, and statistical considerations (NCRP, 2018). These considerations will be discussed in detail later in the chapter when the use of linear nonthreshold models is considered.

TABLE 43.4 Key human populations studied following brief external exposure to X- or gamma radiation.

Population	Effect	Key references
Mixed accident victims	Primarily acute	International Atomic Energy
	radiation sickness	Agency
Atomic bomb survivors	Cancer and other diseases	Ozasa et al. (2012)
Prenatal irradiation	Leukemia	Doll and Wakeford (1997)
	Cancer	
Ankylosing spondylitis patients	Cancer	Court Brown and Doll (1965)
Radiologists	Leukemia	Matanowski et al. (1975)
		Smith and Doll (1981)
Thymic enlargement	Thyroid cancer	Shore et al. (1993)
Tinea capitis	Thyroid cancer	Shore et al. (2003)
		Ron et al. (1989)

TABLE 43.5 Key human populations studied following intake of radioactivity.

Population	Radiation source	Target organ	Key references
Uranium miners	Radon and daughters	Lung	BEIR VI (1999)
Radium dial painters	^{226}Ra and ^{228}Ra	Skeleton	Evans et al. (1972)
			Rowland (1994)
			Taylor (1989)
Thorotrast patients	ThO_2	Liver and spleen	Olsen et al. (1990)
			Taylor (1989)
Thyrotoxicosis patients	^{131}I	Thyroid	Dobyns et al. (1974)
			Ron et al. (1989)
			Holm et al. (1980)
Thyroid diagnosis patients	^{131}I	Thyroid	Holm et al. (1989)
Marshall Islanders	^{131,132,133,135}I	Thyroid	Conrad et al. (1980)
Mayak workers	^{239}Pu	Skeleton	Sokolnikov et al. (2008)

Soon after the USAEC was created, the agency recognized there were critical voids in knowledge of radiation effects that could be best addressed with lifespan studies in multiple species, including rodents and dogs. A review of this extensive research is beyond the scope of this chapter. Thus, I will focus on the dog studies that have provided useful information in complementing the results of epidemiological studies. These are shown in Table 43.6.

As already noted, the principal product of the Hanford site was ^{239}Pu. Thus, when a biological research program was initiated there, one of the major research efforts focused on the health effects of ^{239}Pu. The inhalation route of exposure was used since this was considered a likely route of accidental occupational exposures. Later, studies with ^{238}Pu were initiated as concern arose for its use as a thermal source in SNAP devices.

The University of Utah program focused on the critical issue of estimating the cancer health hazards of alpha particle-emitting ^{239}Pu building on earlier data from the radium dial painters who ingested ^{226}Ra,

TABLE 43.6 Major lifespan studies with internally deposited radionuclides in Beagle dogs with multiple exposure levels and lifespan observation.

Laboratory	Radionuclides	Route of administration
Hanford	$Pu^{238}PuO_2$, $Ra^{239}O_2$, Pu^{239}, nitrate	Inhalation of polydisperse aerosols
University of Utah	Ra^{226}, Ra^{228}, Th^{228}, Pu^{239}, Am^{241}, Sr^{90}	Single intravenous injection
UC Davis	Ra^{226}	Multiple intravenous injections
	Sr^{90}	Chronic ingestion
Lovelace	Y^{90}, Y^{91}, Ce^{144} and Sr^{90} Chloride	Inhalation of polydisperse aerosol
	$Cs^{137}Cl_2$	Intravenous injection
	Y^{90}, Y^{91}, Ce^{144} and Sr^{90} in fused aluminosilicate particle	Inhalation of polydisperse aerosols

^{228}Ra, and ^{230Th}, all of which had alpha particle emissions. Intravenous injections were used to administer the radionuclides. Dogs were used instead of rats because the distribution of administered Pu in dogs (i.e., liver to skeleton ratios) was similar to that found in humans. Rats were not viewed as a good model for humans. As increased concern developed in the 1950s for the effects of nuclear weapons testing and fallout ^{90}Sr, a beta particle-emitting radionuclide on children, a decision was made to enlarge the Utah program to include dogs given single injections of ^{90}Sr.

Yet later a decision was made to create a new program at the University of California-Davis in which dogs were fed ^{90}Sr. Female dogs were mated, ingested ^{90}Sr, and nursed their pups exposing them to ^{90}Sr via their dam's milk. The pups ingested ^{90}Sr until they were 540 days old. Other dogs received six injections of ^{226}Ra to link the University of California-Davis results to the radium dial painters and the Utah studies. All the dogs were followed for their lifespan.

The Lovelace Fission Product Inhalation Program, later renamed the Inhalation Toxicology Research Institute, was initiated in 1960 in response to increasing concern over the potential for a catastrophic nuclear reactor accident. An AEC Report (WASH-740) prepared by Brookhaven National Laboratory (BNL, 1957), highlighted deficiencies in the information available to estimate the hazards of airborne releases of gamma ray- and beta particle-emitting fission product radionuclides in the event of a catastrophic reactor accident. Later the Lovelace program was expanded to include research on inhaled ^{239}Pu as the potential for use of ^{239}Pu as a breeder reactor fuel was being evaluated. Likewise, ^{238}Pu was added to the Lovelace Program as it became the preferred fuel for SNAP devices. A unique feature of the Lovelace Program was the use of monodisperse particles of ^{238}Pu and ^{239}Pu.

The results of the Beagle studies have been reported in hundreds of peer-reviewed publications. In this chapter I will selectively summarize some of the results focusing on cancer induction. These studies are reviewed in greater detail in McClellan (2014, 2019).

It is important to note that all of the studies conducted in Beagles involved the conduct of toxicokinetic studies linked to the lifespan studies (Fig. 43.9). Thus, it was possible to provide an estimated radiation dose pattern for multiple potential target organs for each individual dog. Each dog was followed closely throughout its life and at death, detailed necropsies were conducted, and causes of death identified. Thus, a vast array of data were available for evaluation of radiation–dose response relationships. Indeed, the data were made available publicly and have been used by scientists around the world. The data, as well as some tissues, from the Lovelace studies are held in a repository at Northwestern University.

Two critical questions were addressed by the dog studies: (1) what is the relative effectiveness of alpha- versus beta-irradiation? and (2) how does dose protraction influence the effectiveness of the internally delivered radiation dose? It is important to recognize that the number of dogs studied in the various studies was not sufficient based on statistical limitations to address the nagging question of whether dose–response relationships at low doses were or were not linear.

43.15.1.1 Overview of key biological mechanisms

Let us now turn our attention to adverse health outcomes resulting from radiation exposures. Before addressing specific health outcomes it is appropriate to emphasize that exposure–dose–health response relationships for ionizing radiation are strongly influenced by the quality of the radiation and by both dose rate and total dose as a

function of time. Both fractionation and protraction of the delivery of the radiation dose will reduce the health impact per unit of delivered radiation dose. The influence of these factors is schematically illustrated in Fig. 43.10. The panel on the left illustrates the situation for most external exposures. The time interval for exposure and delivered dose coincide. This is the situation for external radiation from detonation of a nuclear weapon or a single treatment session for a cancer patient receiving external beam therapy. The panel on the right illustrates the situation for intake of radioactive material. The intake via inhalation or ingestion may be brief. The delivered radiation dose to various tissues from ingestion or inhalation of radionuclides will be protracted and the radiation dose pattern dependent on the distribution of the radioactive material in the body determined by biological processes and the physical half-life of the radionuclide.

As an aside, one of the first radiation effects studies I conducted compared the effects of ^{131}I and X-irradiation on the sheep thyroid (McClellan et al., 1963). The radiation dose pattern from the X-irradiation was analogous to that shown in the left panel of Fig. 43.10, while the ^{131}I yielded protracted beta-irradiation was analogous to the pattern shown on the right. The X-irradiation was substantially more effective than the protracted dose from ^{131}I in producing early injury to the thyroid.

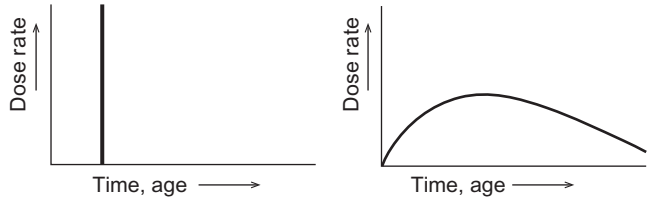


FIGURE 43.10 External brief radiation exposures and resulting tissue dose occur simultaneously while the intake of radioactivity results in a protracted tissue dose.

The effects of radiation on living things begin with interactions at the molecular and cellular levels (recall Fig. 43.3). The interactions begin with ionization or excitation of orbital electrons and results in the deposition of energy in cells. The ionizations can directly affect biological molecules, principally DNA. In addition, there may be indirect action of radiation mediated by free radicals that can diffuse far enough to reach other target molecules. This is noteworthy since the body is about 80% water. When atoms are ionized, their chemical binding characteristic changes. The ionization may result in breakage of molecules or relocation of atoms within molecules. These changes, in turn, can alter cell function, including causing cell death or mutations in surviving cells. Ionization of water or biological molecules is an initial step in the complex processes illustrated in Fig. 43.3. Many of these processes are described in the 100 most-cited papers published in the *Radiation Research Journal*. The textbooks by Bushong (2017), Hall and Giaccia (2012), Mettler and Upton (2008), and Turner (2007) all provide in-depth coverage of the vast literature on mechanisms of action of ionizing radiation.

Ionization in cells results in cell death, mutations, and other alterations in cell function. Cell killing and functional alterations in surviving cells can alter organ function and with sufficiently high radiation doses lead to death of those exposed individuals. The most common form of cell death from radiation is mitotic death. The irradiated cells die as they attempt to divide because of the severe damage to their chromosomes. This is the basis of the effectiveness of radiation in treating cancer. An example of the survival of cells related to delivered dose of X-rays is shown in Fig. 43.11. Irradiation of cells produces mutational changes as illustrated in Fig. 43.12. Later, experiments conducted by Brooks (2012) demonstrated the relative biological effectiveness of beta/gamma irradiation versus alpha particles and alpha particles plus

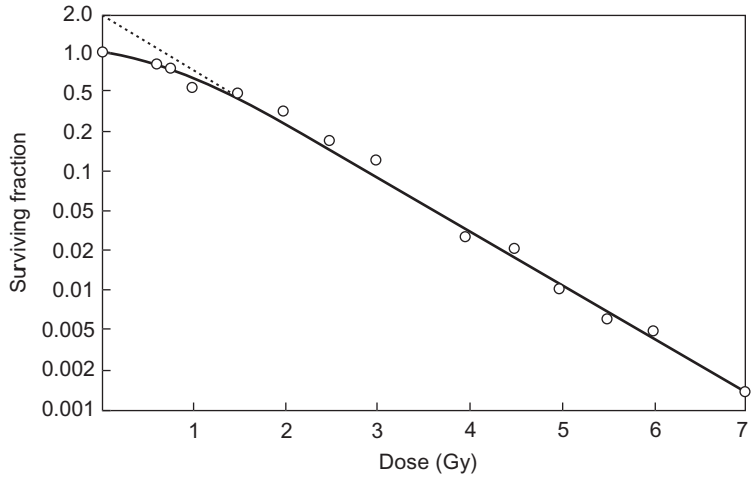


FIGURE 43.11 Survival curve for HeLa cells in culture exposed to X-rays (Puck and Markus, 1956).

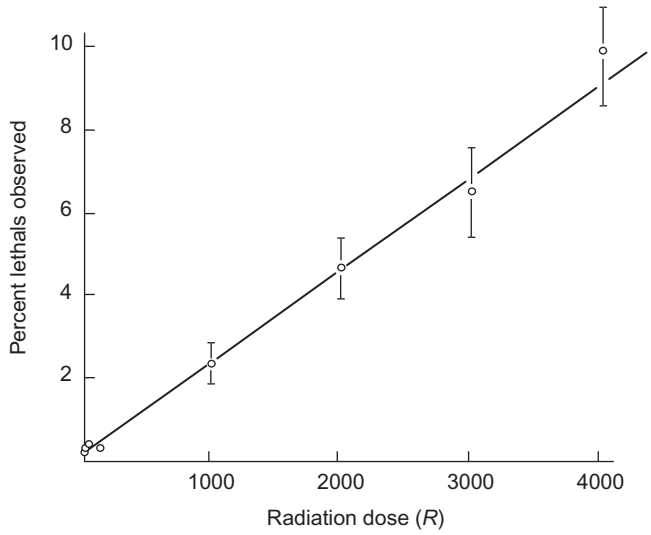


FIGURE 43.12 Dose–response relationship for induction of mutations in X-irradiated *Drosophila*. Note the substantial radiation doses studied. *Adapted from Oliver, C.P., 1930. The effect of varying the duration of x-ray treatment upon the frequency of mutation. Science 71:44-46 (Oliver, 1930).*

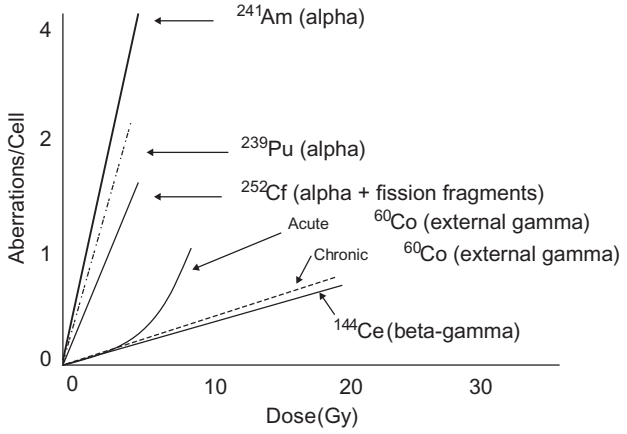


FIGURE 43.13 Dose–response relationship for in vivo induction of chromosome aberrations in liver cells of Chinese hamsters by high LET (alpha particles from $Am^{241,}$ ^{239}Pu, and Cf^{252}) and low LET (CO^{60} gamma rays and Ce^{144}-Pr^{144} beta particles) (Brooks, 2012).

fission fragments (Fig. 43.13). The figure also illustrates the sparing effect, that is, reduced mutations, when ^{60}Co external radiation is protracted.

The postulated role of cell killing and induction of transformations in causing leukemia is illustrated in Fig. 43.14. The critical interplay between cell survival and mutational events and subsequent cell transformation in the induction of cancer was explicitly incorporated in the now classic model developed by Moolgavkar and Venzon (1979). See also Moolgavkar and Knudson (1981), Moolgavkar (1986), Moolgavkar et al. (1988, 1999), and Dewanji et al. (1989).

43.15.2 Acute radiation syndrome and early effects

The acute effects of external radiation, including lethality, have been studied in many different species including humans. Mettler and Upton (2008) estimated the acute radiation dose required to kill 50% of individuals within 60 days. For 10 nonhuman species, in the absence of medical intervention, the LD_{50} (60 days) ranged from 3–5 to 4.0 Gy air or surface dose, corresponding to midline tissue doses of about 2.7 Gy. The range of values is remarkably narrow considering the studies were done at different times with different radiation sources and with subjects maintained under different environmental conditions, diets, and microbial flora.

Early literature on acute effects referred in a generic manner to early deaths being due to multiple acute radiation syndromes, including the nervous, gastrointestinal, or hematopoietic systems. More recent literature uses the term acute radiation sickness (ARS) (Mettler and Upton, 2008). The word acute relates to deaths occurring within a few months of exposure to external radiation or a protracted dose over several months received from intake of a substantial amount of radioactive material. The use of syndrome or sickness refers to the injury occurring in neurovascular, gastrointestinal, hematopoietic, and cutaneous tissues that cause death at various absorbed radiation doses.

Many thousands of individuals died of ARS following the bombings of Hiroshima and Nagasaki, Japan. As discussed earlier, these individuals were also impacted by the blast wave and thermal radiation. These were the individuals who had been exposed to blast and thermal radiation at levels that did not produce immediate lethality.

To give a clear picture of radiation effects alone, it is most appropriate to turn to a unique data set developed by the International Atomic Energy Agency (IAEA) (1998) for evaluating early injury from radiation. This is a registry that includes about 400 cases of which about 100 died of ARS. The other 300 individuals survived despite having some signs and symptoms of ARS. Tables 43.7–43.9 provide useful summaries of the radiation dose-related signs and symptoms of ARS for humans.

As I noted in an earlier review of biomarkers of exposure and effects of ionizing radiation (McClellan, 2019), the biomarkers most extensively studied represent good old-fashioned, tried and proven medical practices used in the diagnosis and treatment of diseases. Since radiation-induced effects were first observed a century ago there has been a search for new biomarkers of radiation-induced effects, a search for "special biomarkers." That research effort was rejuvenated following the September 11, 2001, disaster in New York City as concern was renewed over potential terrorist events, including devices

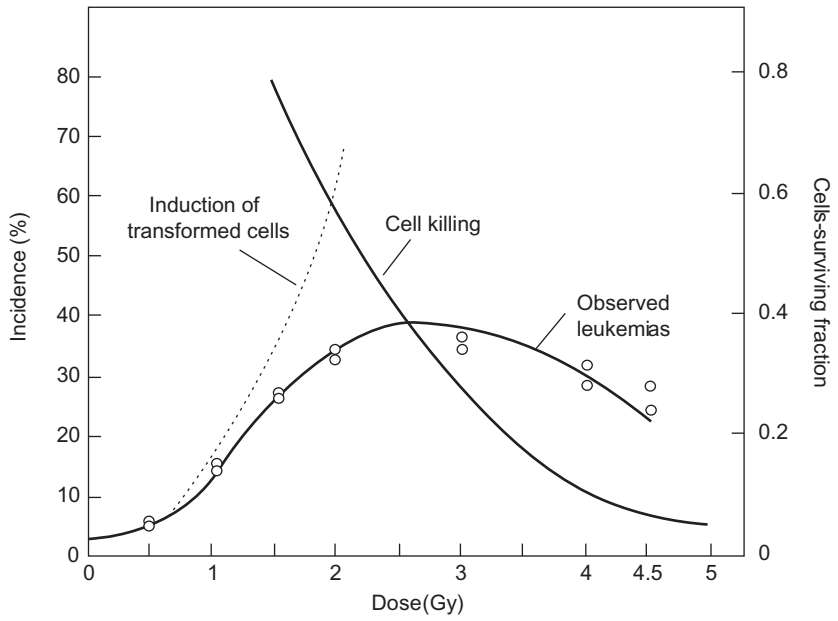


FIGURE 43.14 The postulated role of cell killing and induction of cell killing in producing leukemia in irradiated mice (Gray, 1965).

resulting in radiation exposure. Some of the most recent research findings were reviewed in Ray et al. (2014).

Let me now transition from the health effects of brief external radiation exposures from external sources to the effects of internally deposited radionuclides. At the outset, I note that there are limited data from human studies on the acute effects of internally deposited radionuclides. This is the case because it is difficult to create situations in which the intake of radioactive materials is sufficient to result in radiation doses that will result in acute radiation-induced effects. I have underlined this statement to give emphasis to this point for any policy makers or potential terrorists reading this chapter.

There is one case in which a postulated single intake of radioactive material resulted in an acute death of a human and was a purposeful poisoning with ^{210}Po. Another case involved the accidental treatment of a patient with an erroneously high dose of a radio- therapeutic agent, ^{198}Au.

43.16 Early radiation effects from internally deposited radionuclides

In the absence of human experience with internally deposited radionuclides and acute injury, it is useful to examine the data from studies with laboratory animals (Table 43.6). In my view the most relevant data for describing the acute effects of internally deposited radionuclides are from studies conducted by Lovelace scientists. In the studies, Beagles were exposed briefly (on the order of an hour) to graded concentrations of various fission product radionuclides. It was originally planned to use inhalation exposures for all the studies. This included giving dogs ^{137}CsCl by inhalation, however, a few calculations indicated the exposure conditions could pose a hazard to the personnel conducting the inhalation exposures. Thus, recognizing that inhaled ^{137}CsCl and intravenously injected ^{137}CsCl are distributed in the same manner in the body, resulting in a similar radiation dose pattern, animals were exposed by intravenous injection.

Using different radionuclides with varying physical half-lives, the radiation dose patterns realized in the several studies were quite different (Table 43.10). With large intakes of the fission product radionuclides, the resulting radiation doses to hematopoietic tissues were sufficient to cause lethality in weeks to a few months from hematopoietic injury. As illustrated in Fig. 43.15, the protracted radiation dose from injected ^{137}CsCl or inhaled $^{91}YCl_3$, $^{90}SrCl_2$, or $^{144}CeCl_3$ was about one-fourth as effective as a brief exposure to 1 MeV X-rays in producing the acute bone marrow syndrome in dogs.

Studies were also conducted in which Beagles briefly inhaled ^{90}Y-, ^{91}Y-, ^{144}Ce-, or ^{90}Sr-infused aluminosilicate particles (FAPs). In these studies, the FAPs served as a particulate matter vehicle. The difference in radiation dose pattern for the different beta particle-emitting radionuclides was determined by differences in their physical half-lives. These exposures resulted in protracted radiation doses to the respiratory tract. At the highest doses to the lungs, in excess of 30 Gy, pneumonitis developed producing death within about 500 days of the initial intake of the radioactive aerosol (Fig. 43.16). For comparative purposes, human data are shown for pneumonitis induction by brief exposure to X-rays. The protracted low-LET irradiation in the dogs is substantially less effective in

TABLE 43.7 Prodromal phase of acute radiation sickness (ARS).

Degree of ARS and approximate dose of Acute WBE (Gy)					
Symptoms and medical response	**Mild (1–2 Gy)**	**Moderate (2–4 Gy)**	**Severe (4–6 Gy)**	**Very Severe (6–8 Gy)**	**Lethal[a] (>8 Gy)**
Vomiting onset	2 h after exposure or later	1–2 h after exposure	Earlier than 1 h after exposure	Earlier than 30 min after exposure	Earlier than 10 min after exposure
% of incidence	10–50	70–90	100	100	100
Diarrhea	None	None	Mild	Heavy	Heavy
Onset	–	–	3–8 h	1–3 h	Within min or 1 h
% of incidence	–	–	<10	>10	Almost 100
Headache	Slight	Mild	Moderate	Severe	Severe
Onset	–	–	4–24 h	80	1–2 h
% of incidence	–	–	50	May be altered	80–90
Consciousness	Unaffected	Unaffected	Unaffected		Unconsciousness (may last s/min)
Onset	–	–	–	–	S/min
% of incidence	–	–	–	–	100 (at >50 Gy)
Body temperature	Normal	Increased	Fever	High fever	High fever
Onset	–	1–3 h	1–2 h	<1 h	<1 h
% of incidence	–	30–80	80–100	100	100
Medical response	Occupation observation	Observation in general hospital, treatment in specialized hospital if needed	Treatment in specialized hospital	Treatment in specialized hospital	Palliative treatment (symptomatic only)

WHE: whole body exposure.
[a]*With appropriate supportive therapy individuals may survive for 6–12 months with whole-body doses as high as 12 Gy.*
Source: Adapted from International Atomic Energy Agency (IAEA). Diagnosis and treatment of radiation injuries. Safety report series No. 2, Vienna, IAEA, 1998.

inducing injury than less protracted irradiation. The more extended or protracted the delivery of the radiation dose, the larger the integrated dose required to produce equivalent effects. Dose rate and dose protraction are very important!

It is important to note that the cumulative radiation doses required to produce radiation-induced effects from intake of radioactive materials are on the order of 10 Gy and higher for hematopoietic injury and 50 Gy and higher for pneumonitis. The air concentrations of radioactive materials and exposure durations required to produce these radiation doses are very substantial!

It is also important to recognize that special effort was taken to produce the experimental highly respirable aerosols used in the research described above. The aerosols had an activity median aerodynamic diameter on the order of 1–2 μm, a size selected to minimize nasopharyngeal deposition and maximize pulmonary deposition. Most crude devices that might be used to create radioactive aerosols to purposefully harm people will produce larger particles that are not as readily respired and deposited in the respiratory tract.

In closing this section, let me briefly address the issue of potential treatments to reduce radiation doses from internally deposited radionuclides. The US Centers for Disease Control and Prevention maintain a website on radiation emergencies (CDC, 2019). It provides general guidance for dealing with radiation emergencies. This includes the use of stable iodine to minimize the uptake and radiation dose of ^{131}I (recall Fig. 43.8). This topic is also covered in detail in a special report by the World Health Organization (WHO, 1999).

In my opinion, there are no other treatments that can realistically be used to treat populations numbering in the

TABLE 43.8 **Latent phase of acute radiation sickness (ARS).**

	Degree of ARS and approximate dose of acute WBE (Gy)[a]				
	Mild (1–2 Gy)	**Moderate (2–4 Gy)**	**Severe (4–6 Gy)**	**Very Severe (6–8 Gy)**	**Lethal (>8 Gy)**
Lymphocytes (G/L) (days 3–6)	0.8–1.5	0.5–0.8	0.3–0.5	1.0-0.3	0.0–0.1
Granulocytes (G/L)	>2.0	1.5–2.0	1.0–1.5	≤ 0.5	≤ 0.1
Diarrhea	None	None	Rare	Appears on days 6–9	Appears on days 4–5
Epilation	None	Moderate beginning on day 15 or later	Moderate, beginning on days 11–21	Complete earlier than day 11	Complete earlier than day 10
Latency period (days)	21–35	18–28	8–18	7 or less	None
Medical response	Hospitalization not necessary	Hospitalization recommended	Hospitalization necessary	Hospitalization urgently necessary	Symptomatic treatment only

[a]*G/L gigaliter. WBE, whole-body exposure.*
Source: Adapted from International Atomic Energy Agency (IAEA). Diagnosis and treatment of radiation injuries. Safety report series No. 2, Vienna, IAEA, 1998.

TABLE 43.9 **Findings of critical phase of acute radiation sickness (ARS) following whole-body exposure (WBE).**

	Degree of ARS and approximate dose of acute WBE (Gy)[a]				
	Mild (1–2 Gy)	**Moderate (2–4 Gy)**	**Severe (4–6 Gy)**	**Very Severe (6–8 Gy)**	**Lethal (>8 Gy)**
Lymphocytes (G/L)	0.8–1.5	0.5–0.8	0.3–0.5	0.1–0.3	0.0–0.1
Platelets (G/L)	60–100	10–60	25–35	15–25	<20
	10%–25%	25%–40%	40%–80%	60%–80%	80%–100%[b]
Clinical manifestations	Fatigue, weakness	Fever, infections, bleeding, weakness, epilation	High fever, infections, bleeding, epilation	High fever, diarrhea, vomiting, dizziness and disorientation, hypotension	High fever, diarrhea, unconsciousness
Lethality (%)	0	0–50	20–70	50–100	100
		Onset 6–8 wk	Onset 4–8 wk	Onset 1–2 wk	1–2 wk
Medical response	Prophylactic	Special prophylactic treatment from days 14–20; isolation from days 10–20	Special prophylactic treatment from days 7–10; isolation from the beginning	Special treatment from the first day; isolation from the beginning	Symptomatic only

[a]*1 Gy = 100 rads.*
[b]*In very severe cases, with a dose >50 Gy, deaths precedes cytopenia.*
Source: Adapted from International Atomic Energy Agency (IAEA). Diagnosis and treatment of radiation injuries. Safety report series No. 2, Vienna, IAEA, 1998.

TABLE 43.10 The physical and effective half-lives and the length of time required for deposition of 90% of the total dose.

Radionuclide-infused aluminosilicate particles	Physical half-life	Effective half-life in lung (9 d)	Time to deliver 90% of total dose
^{90}Sr	29 yr	600	5.5 yr
^{144}Ce	285 d	175	1.6 yr
^{91}Y	59 d	50	0.5 yr
^{90}Y	2.6 d	2.5	8 d

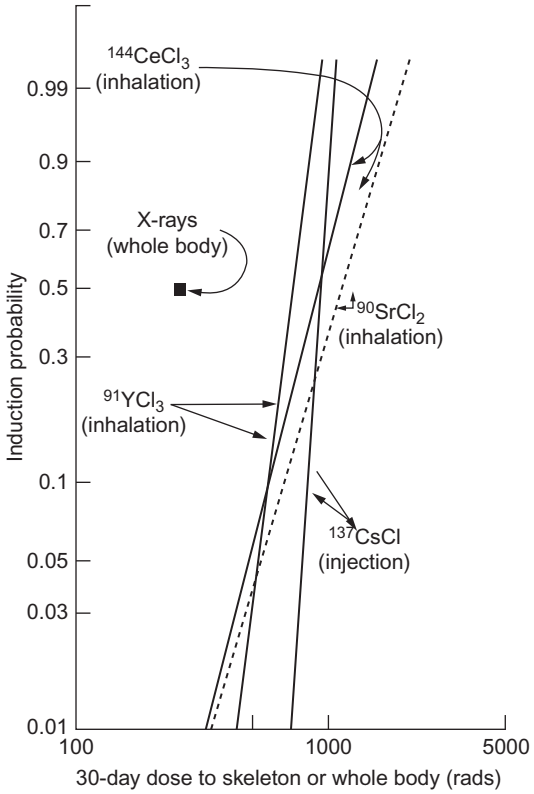


FIGURE 43.15 Decreased effectiveness of protracted internal beta irradiation from soluble forms of ^{91}Y, ^{144}Ce, or ^{90}Sr inhaled or injected ^{137}Cs in producing deaths due to hematopoietic injury (Scott, 1979).

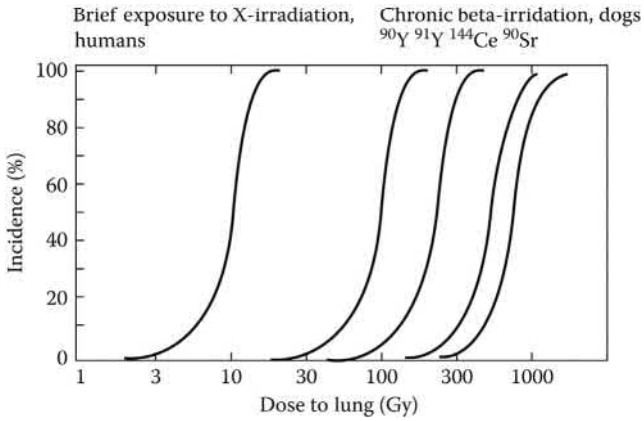


FIGURE 43.16 Decreased effectiveness of protracted internal beta irradiation compared to very brief external X-irradiation in producing pneumonitis (Scott et al., 1988).

thousands to either reduce their radiation dose or treat radiation-induced injury after a nuclear detonation.

43.16.1 Radiation-induced cancer in humans from acute exposures

At this juncture, it is appropriate to discuss the late-occurring effects of radiation exposure. In my opinion, the most relevant data set for detailed analysis are the Japanese A-bomb survivors. This population was originally assembled under the auspices of the Atomic Bomb Casualty Commission (ABCC), now reconstituted as the Radiation Effects Research Foundation (RERF). This research has been supported over the decades by the governments of Japan and the United States. There are three ABCC/RERF cohorts. The Life Span Study (LSS) cohort includes 120,321 individuals; 82,214 in Hiroshima and 38,107 in Nagasaki. A second cohort includes about 3300 individuals who received their exposure in utero as a result of their mother's whole-body exposure. Information from this cohort has provided valuable insights into the incidence of malformations, growth retardation, microcephaly, and mental retardation related to radiation exposure. A third cohort consists of 77,000 children born to the survivors of the atomic bombings. Observation of this latter cohort has provided valuable data for assessing heritable effects (Neel et al., 1990; Satoh et al., 1996). Grant et al. (2015) reported on the risk of death among children of atomic bomb survivors.

The first report on mortality in the LSS cohort was published by Beebe et al. (1962). With each report the duration of follow-up is increased. The 14th report in this

series was published by Ozasa et al. (2012, 2013). A recent paper by Grant et al. (2015) reviews follow-up from 1958 through 2009.

The basic dose-related characteristics of the LSS population are shown in Table 43.11. It is of interest to note that of the 86,601 individuals enrolled in the cohort in 1950, a total of 46,614 individuals (53.8%) had died by 2003, with 39,987 (46.2%) surviving. A total of 10,929 had died with solid cancers with an estimated 527 cancer deaths attributed to radiation exposure using statistical methods. None of these 527 radiation-attributable cases carried a sign "Caused by Cancer." Most people, including many scientists, reviewing these data are surprised at the relatively few cancers attributed to radiation exposure. Many people, including scientists, consider radiation to be a super carcinogen. In fact, radiation is not a very effective carcinogen. Look at the data!

A detailed presentation of the array of specific causes of death is given in Fig. 43.17.

Note the data are shown as excess relative risk (ERR) per Gy. Many experimentalists will not be familiar with the analytical approach using baseline risk and estimated ERR to describe effects. Most experimentalists customarily present absolute incidence values for controls and toxicant-exposed groups. Indeed, many rat bioassays are truncated at 2 years. This has traditionally been done to make it easier to distinguish toxicant-induced cancers from spontaneous cancers in controls with all animals entered in an experiment at 6–12 weeks of age and observed for 2 years. The low incidence of cancers observed in controls by about 2 years of death presumably makes it easier to detect an increase in cancer related to the toxicant exposure, the presence of a cancer hazard. Unfortunately, in my opinion, this well-intentioned approach is now outdated. It was useful in identifying agents that pose a significant carcinogenic hazard to rodents as a harbinger of carcinogenic hazards to humans. Unfortunately, this approach is not very useful in helping understand cancer induction late in life when low exposures (doses of toxic agents) may cause small increases in cancer risk on top of substantial spontaneous incidence. Small increases in cancer associated with administration of toxicants may very likely involve the same mechanisms operative in producing the background incidence of cancers.

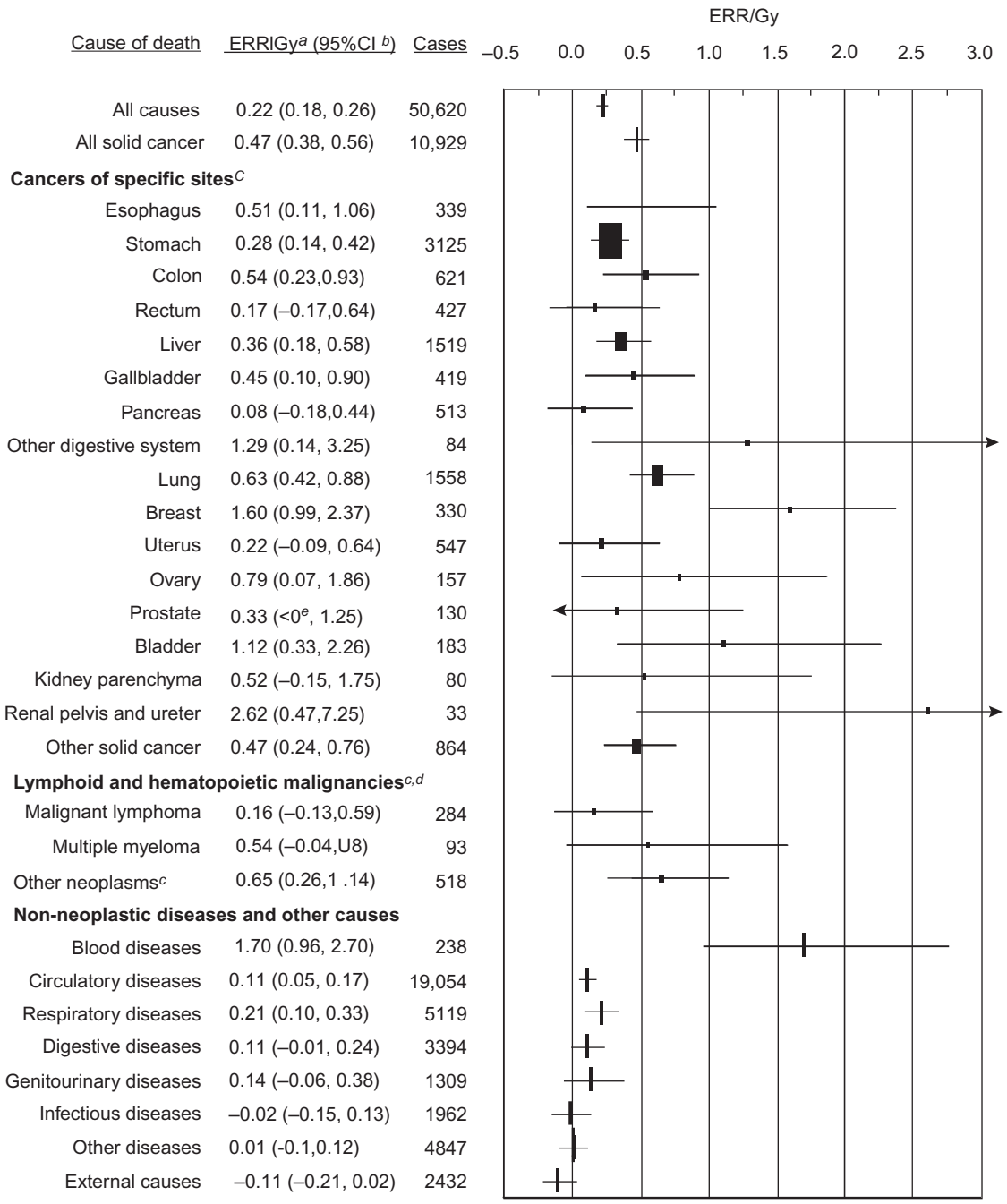
The influence of radiation dose on induction of solid cancers in the LSS cohort is shown graphically in Fig. 43.18. Note the arithmetic horizontal scale. In this presentation, it appears there is a linear relationship between radiation dose and ERR. The same data analyzed by a different team of investigators are presented in Fig. 43.19. Note that a logarithmic scale is used in this figure. With the latter mode of presentation, it appears that a threshold exists in the radiation dose–ERR relationship.

Recall the issue of alternative dose–response relationships raised by Henshaw (1941) and illustrated earlier in Fig. 43.5. The debate over the choice of radiation dose–response relationship models continues three-quarters of a century after the debate was framed. Edward

TABLE 43.11 Observed and estimated excess deaths in cancer and noncancer diseases in the ABCC/RERF life span study (LSS) cohort.

			Solid cancer			Noncancer diseases[b]		
Colon dose (Gy)	Number of subjects	Person-years	Number of deaths	Number of excess cases[a]	Attributable fraction (%)	Number of deaths	Number of excess cases[b]	Attributable fraction (%)
<0.005	38,509	1,465,240	4,621	2	0	15,906	1	0
0.005-	29,961	1,143,900	3,653	49	1.3	12,304	36	0.3
0.1-	5,974	226,914	789	46	5.8	2,504	36	1.4
0.2-	6,356	239,273	870	109	12.5	2,736	82	3.0
0.5-	3,424	129,333	519	128	24.7	1,357	86	6.3
1-	1,763	66,602	353	123	34.8	657	76	11.6
2 +	624	22,947	124	70	56.5	221	36	16.3
Total	86,611	3,294,210	10,929	527	4.8	35,685	353	1.0

[a]*Based on the ERR model was defined as the linear model with effect modification:* $\gamma_0(c,s,b.a)[1+\beta_1 d \bullet exp(\tau e+\upsilon\ 1\ n(a)) \bullet (1\ \sigma\ s)]$.
[b]*Non-neoplastic blood diseases were excluded from noncancer diseases.*
Source: Ozasa et al. "Studies of the mortality of atomic bomb survivors, report 14, 1950–2003: An overview of cancer and noncancer diseases," *Radiat. Res.* 177 (2012) 229–243.



Cause of death	ERRIGy[a] (95%CI [b])	Cases
All causes	0.22 (0.18, 0.26)	50,620
All solid cancer	0.47 (0.38, 0.56)	10,929
Cancers of specific sites[C]		
Esophagus	0.51 (0.11, 1.06)	339
Stomach	0.28 (0.14, 0.42)	3125
Colon	0.54 (0.23,0.93)	621
Rectum	0.17 (–0.17,0.64)	427
Liver	0.36 (0.18, 0.58)	1519
Gallbladder	0.45 (0.10, 0.90)	419
Pancreas	0.08 (–0.18,0.44)	513
Other digestive system	1.29 (0.14, 3.25)	84
Lung	0.63 (0.42, 0.88)	1558
Breast	1.60 (0.99, 2.37)	330
Uterus	0.22 (–0.09, 0.64)	547
Ovary	0.79 (0.07, 1.86)	157
Prostate	0.33 (<0[e], 1.25)	130
Bladder	1.12 (0.33, 2.26)	183
Kidney parenchyma	0.52 (–0.15, 1.75)	80
Renal pelvis and ureter	2.62 (0.47,7.25)	33
Other solid cancer	0.47 (0.24, 0.76)	864
Lymphoid and hematopoietic malignancies[c,d]		
Malignant lymphoma	0.16 (–0.13,0.59)	284
Multiple myeloma	0.54 (–0.04,U8)	93
Other neoplasms[c]	0.65 (0.26,1 .14)	518
Non-neoplastic diseases and other causes		
Blood diseases	1.70 (0.96, 2.70)	238
Circulatory diseases	0.11 (0.05, 0.17)	19,054
Respiratory diseases	0.21 (0.10, 0.33)	5119
Digestive diseases	0.11 (–0.01, 0.24)	3394
Genitourinary diseases	0.14 (–0.06, 0.38)	1309
Infectious diseases	–0.02 (–0.15, 0.13)	1962
Other diseases	0.01 (-0.1,0.12)	4847
External causes	–0.11 (–0.21, 0.02)	2432

FIGURE 43.17 Estimates of excess relative risk (ERR) per Gy and 95% CI for major causes of death. [a]ERR was estimated using the linear dose model, in which city, sex, age at exposure, and attained age were included in the background rates, but not allowing radiation effect modification by those factors. [b]Confidence interval. Horizontal bars show 95% confidence intervals. [c]The size of plots for site-specific cancers was proportional to the number of cases. [d]ERR (95% CI) of leukemia was 3.1 (1.8, 4.3) at 1 Gy and 0.15 (-0.01, 031) at 0.1 Gy based on a linear-quadratic model with 318 cases (not displayed in the figure). [e]The lower limit of 95% CI was lower than zero, but not specified by calculation (1950−2003) (Ozasa et al., 2012).

Calabrese has published extensively on radiation hormesis and its implications for risk assessment (Calabrese and Baldwin, 2000, 2003; Calabrese, Baldwin and Holland, 1999; Calabreese, 2011). He has also raised questions concerning ethical issues surrounding the early radiation assessments concerning effects at low doses (Calabrese, 2011).

A strength of the ABCC/RERF data set is that the individuals were all exposed in the same manner, a single brief exposure to external radiation. Thus, the use of the

data is most relevant to evaluating the effects of acute radiation doses and setting standards to limit such exposures.

43.16.1.1 Radiation-induced cancer from internally deposited radionuclides

This naturally leads to a question as to the availability of data on chronic exposures to radiation, including chronic exposures from internally deposited radionuclides. As noted earlier, some human data on this topic are available (Table 43.5). However, there are challenges involved in interpreting each of them.

In my opinion, the results of the dog studies are particularly useful in understanding the adverse effects of chronic irradiation from internally deposited radionuclides. A comparison of the effectiveness of protracted beta irradiation of the lung in producing lung cancer is shown in Table 43.12. Recall from Table 43.10 that ^{90}Y in FAP delivered 90% of its total dose in 8 days, whereas ^{90}Sr in FAP delivered 90% of its total dose in 5.5 years. The longer protraction of dose delivery for ^{90}Sr in FAP resulted in it being only 0.22% as effective as ^{90}Y in producing cancer. The two radionuclides, ^{91}Y and ^{144}Ce, with intermediate physical half-lives, had potency values intermediate between those for ^{90}Y in FAP and ^{90}Sr in FAP.

These data are shown in a schematic presentation in Fig. 43.20. In addition, this figure illustrates the greater effectiveness of chronic alpha irradiation of the lung from ^{239}Pu oxide compared to chronic beta irradiation in inducing lung cancer. The comparative effectiveness of alpha irradiation of bone from ^{226}Ra, ^{228}R, ^{241}Am, ^{238}Pu, and ^{239}Pu compared to beta irradiation from ^{90}Sr in inducing bone cancer is also evident in Fig. 43.20. The comparative potency of several different alpha particle emitters versus beta irradiation from ^{90}Sr in inducing bone cancer is evident from the results shown in Table 43.13. Of the various alpha emitters, the volume-seeking ^{226}Ra and ^{238}Ra are less effective than the bone surface-seeking Pu.

The comparative effectiveness for Pu inhaled in several forms in inducing lung cancer is shown in Table 43.14. ^{239}Pu nitrate was more effective in inducing lung cancer than the other forms of Pu. This is postulated to be related to the ^{239}Pu nitrate distributing more uniformly in the lung and being more effective in damaging lung cells than particulate Pu oxide. The results from the Lovelace studies with inhaled monodisperse particles are of special interest. The smaller the particles, the greater the total number of particles inhaled with equal total quantities of radioactivity. Thus, for equal radioactivity, more 0.75-μm particles will be deposited than 1.5-μm particles and even fewer 3.0-μm particles. For induction of

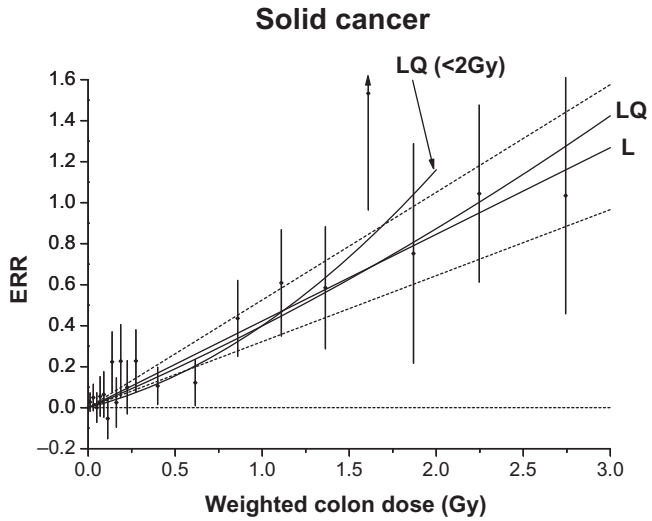


FIGURE 43.18 Excess relative risk (ERR) for all solid cancers in relation to radiation exposure. The black circles represent ERR and the bars are the 95% confidence intervals for the dose categories together with trend estimates based on linear (L) with 95% confidence intervals (dotted lines) and linear quadratic (L-Q) models using the full dose range and LQ model for data restricted to dose < 2 Gy (Ozasa et al., 2012).

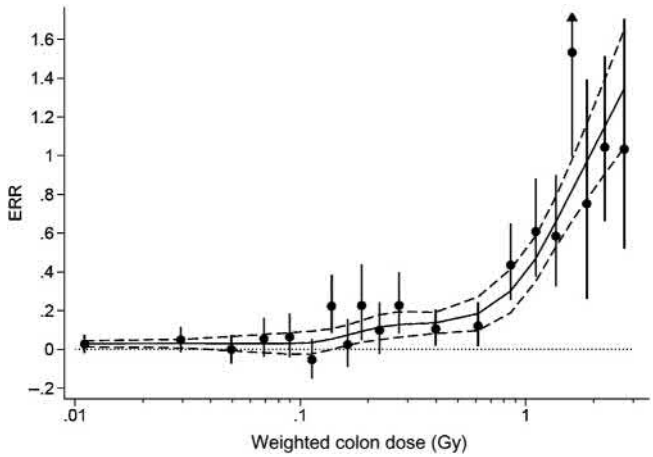


FIGURE 43.19 Excess relative risk (ERR) for all solid cancers in atomic bomb survivors in relation to radiation exposure. The black circles and error bars represent ERR and 95% CIs for the dose categories. Data from Ozasa et al. (2013). Solid line—fit to the ERR data using a multiple linear regression in which weighted log colon dose was entered into the model using a restricted cubic spline transformation with five knots. Regression weights were equal to the inverse of the variance of the point estimates. Dashed lines are 95% CI of the fit. Figure from performing analysis equivalent to Doss et al. (2012) with the corrected data in Ozasa et al. (2013). Figure provided by Brian L. Egleston. (Doss, 2013).

TABLE 43.12 Summary of relative carcinogenic and dosimetric ratios from four beta-emitting radionuclides inhaled in fused aluminosilicate particles and inducing lung cancer.

Nuclide	Emission type	Organ	Laboratory	Potency ratio
^{90}Y	β	Lung	Lovelace ITRI	1.0
^{91}Y	β	Lung	Lovelace ITRI	0.51
^{144}Ce	β	Lung	Lovelace ITRI	0.27
^{90}Sr	β	Lung	Lovelace ITRI	0.22

Source: Raabe, O.G. "Concerning the health effects of internally deposited radionuclides," *Health Phys*. 98, (2010): 515–536.

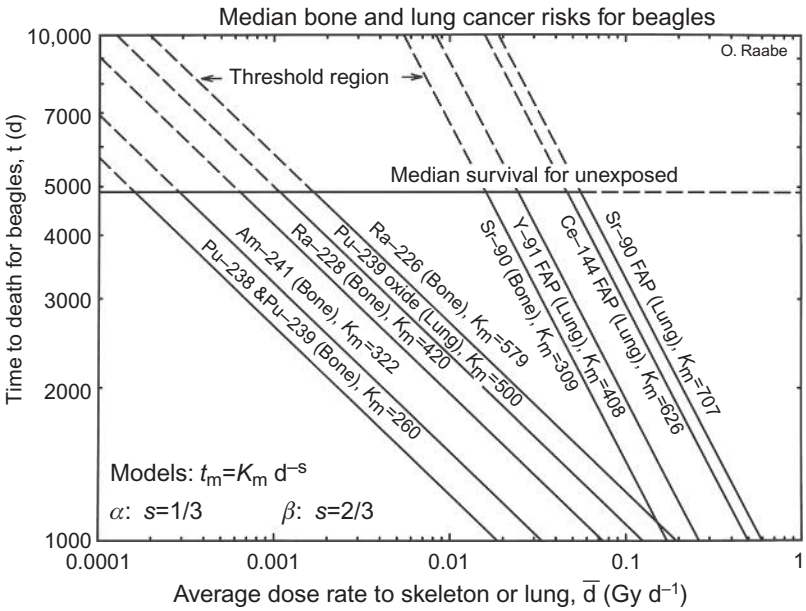


FIGURE 43.20 Illustration of bone sarcoma and lung carcinoma risk functions for Beagles demonstrating similar target organ average-dose-rate/time/response patterns with lifespan virtual thresholds at low dose-rates. The positions of the lines vary because of inherent differences in irradiation of the target cells by the different radionuclides and forms. Data were selected from lifetime laboratory studies of internally deposited radionuclides in Beagles including skeletal deposits of ^{90}Sr after exposure by ingestion at Davis and by injection at Utah, lung deposits of inhaled ^{144}Ce-, ^{91}Y-, and ^{90}Sr-infused aluminosilicate particles (FAP) at ITRI, lung deposits of inhaled $^{239}PuO_2$ at PNL, skeletal deposits of injected ^{226}Ra at Davis and Utah, inhaled $^{238}PuO_2$ at ITRI, and skeletal deposits of injected ^{226}RA and ^{241}Am at Utah (Raabe, 2010).

lung cancer, the 0.75-μm particles were more effective than the 1.5-μm particles, with the 3.0-μm particles least effective. From these summary results, it is apparent that radiation quality (beta versus alpha irradiation), dose protraction, and total dose are very important for cancer causation in the lung or skeleton.

It is appropriate to ask whether the results from the dog studies are relevant to estimating human hazards. The lifespan studies in Beagles were initiated in part because it was thought the dogs would serve as effective surrogates for human populations. At that time, it was not anticipated that human data would become available from worker populations. This proved to be the case in the United States and Britain. The protection goals in the US or British workers were always zero exposure with no intake of ^{239}Pu. The maximum body burden among US and British workers was 3.2 Bq (Wiggs et al., 1994; Voelz et al., 1997; Omar et al., 1998; Brown et al., 2004; Wing et al., 2004). No excess risk of lung, liver, or skeletal cancer was observed. The situation would prove quite different in the case of Russian workers.

With a brief recess in the Cold War in the early 21st century, there was increased scientific cooperation between the United States and the USSR in some areas. One of the areas of cooperation related to access to health data on key populations. One of these populations was the workers at the Mayak Production Association (MPA) facility in Russia which produced and handled large quantities of ^{239}Pu. Sokolnikov et al. (2008), Tokarskaya et al. (2002, 2006), and Gilbert et al. (2013) have reported on MPA workers. This population included 354 cases of lung cancers, 40 cases of liver cancers, and 11 cases of bone cancer. Five of the bone cancers were in controls and six in exposed workers. The small number of bone cancers was a surprise since for decades it was assumed that if an increase in cancers were to be observed in Pu workers, it would be bone cancer. Recall the radium dial painter experience.

TABLE 43.13 Summary of relative carcinogenic and dosimetric potency ratios for bone-seeking radionuclides producing bone cancer.

Nuclide/form	Type	Organ	Laboratory	Potency ratio
^{228}Th	α	Bone	University of Utah	12.4
^{239}Pu	α	Bone	University of Utah	12.0
^{238}Pu	α	Bone	Pacific Northwest Lab	11.8
^{238}Pu	α	Bone	Lovelace ITRI	10.6
^{240}Cf	α	Bone	University of Utah	7.5
^{224}Ra	α	Bone	University of Utah	7.0
^{241}Am	α	Bone	University of Utah	5.9
^{239}Pu	α	Bone	Pacific Northwest Lab	4.5
^{252}Cf	α	Bone	University of Utah	4.4
^{228}Ra	α	Bone	University of Utah	2.7
^{226}Ra	α	Bone	University of Utah	1.1
^{226}Ra	α	Bone	UC Davis	1
^{90}Sr (injection)	β	Bone	University of Utah	1.2
^{90}Sr (ingestion)	β	Bone	UC Davis	1

Source: Raabe, O.G. "Concerning the health effects of internally deposited radionuclides," *Health Phys*. 98, (2010): 515–536.

TABLE 43.14 Summary of relative carcinogenic and dosimetric potency ratios as a function of radiation type for inhaled alpha-emitting radionuclides producing lung cancer.

Nuclide/form	Type	Organ	Laboratory	Potency ratio
$^{239}Pu(NO_3)_4$	α	Lung	Pacific Northwest Lab	1
$^{238}PuO_2$	α	Lung	Lovelace ITRI	0.54
$^{239}PuO_2$ 0.75 μm	α	Lung	Lovelace ITRI	0.48
$^{239}PuO_2$ 1.5 μm	α	Lung	Lovelace ITRI	0.42
$^{239}PuO_2$	α	Lung	Pacific Northwest Lab	0.37
$^{239}PuO_2$ 3 μm	α	Lung	Lovelace ITRI	0.31
$^{238}PuO_2$	α	Lung	Pacific Northwest Lab	0.26

Source: Raabe, O.G. "Concerning the health effects of internally deposited radionuclides," *Health Phys*. 98, (2010): 515–536.

With positive data available from ^{239}Pu workers, an obvious question was how these findings in workers compared to those in dogs that had been exposed to ^{239}Pu. To address this issue, Wilson et al. (2010) analyzed a pooled set of data for dogs that were exposed to ^{238}Pu or ^{239}Pu at either Hanford or Lovelace. The lung cancer mortalities for the MPA workers (354 cases) and the dogs (231 cases) are shown in Fig. 43.21. It is important to note that the worker data were analyzed taking account of their smoking history. Many of the MPA workers were heavy smokers. The dogs did not smoke. The similarity of the results for lung cancer in MPA workers and dogs is striking. Indeed, the MPA worker data clearly validate the use of the dog model used at Hanford and Lovelace to produce data relevant to humans for hazard/risk assessment purposes.

The liver cancer mortality rates for MPA workers (14 controls, 26 exposed) and dogs (7 controls and 39

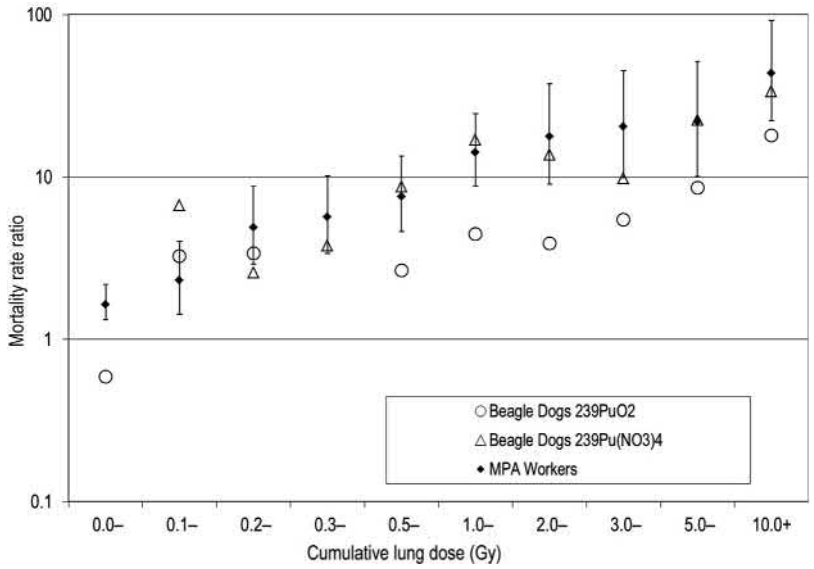


FIGURE 43.21 Lung cancer mortality rate ratio (with 95% confidence intervals) for Mayak Production Association Workers with known plutonium exposure plotted with rate ratios in Beagle dogs exposed by inhalation to plutonium by level of cumulative lung dose (Wilson et al., 2010).

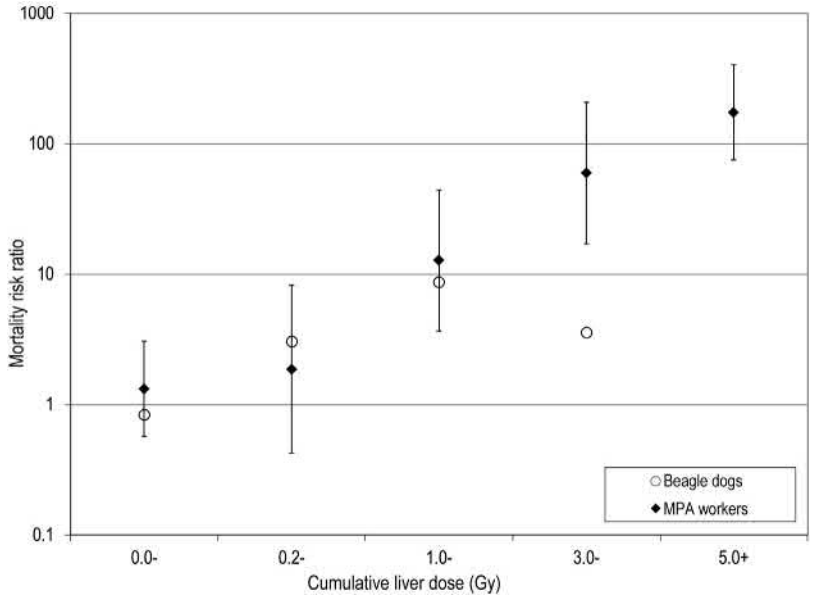


FIGURE 43.22 Liver cancer mortality rate ratios (with 95% confidence intervals) for Mayak Production Association Workers with known plutonium exposure plotted against rate ratios in Beagle dogs exposed to plutonium by level of cumulative liver dose (Wilson et al., 2010).

exposed) are shown in Fig. 43.22. Again, the similarity in the radiation dose–response relationships for workers and dogs is remarkable, especially for liver cases below 3.0 Gy. The lower dose groups included essentially all of the cases in dogs and 14 of 26 cases in workers. Twelve of the liver cancer cases were observed in workers with liver doses of over 3.0 Gy. A key factor to consider in evaluating the liver cancer cases is the important role of alcohol consumption as a confounder in causing liver cancer as pointed out by the Russian investigators (Tokarskaya et al., 2006). The Russian workers at Mayak consumed large quantities of alcohol. The dogs did not consume alcohol.

43.17 Linear nonthreshold models

In a recent report, the National Council on Radiation Protection and measurements (NCRP) evaluated the use of the linear-nonthreshold (LNT) model for radiation protection purposes (NCRP, 2018). In a sense, they addressed the fundamental issue on choice of radiation dose–response relationships posed by Henshaw (1941). The NCRP evaluated 29 different sets of data (Table 43.15). Each of the data sets was independently evaluated with regard to dosimetry, epidemiology, and statistical methods used with a score of 1–3 assigned to each method and then an aggregate score of 1–4 was

TABLE 43.15 Ratings of the quality of cancer studies reviewed with regard to dosimetry, epidemiological statistical methodology, and their aggregate degree of support for the LNT model.

Study (or groups of studies)[a]	Dosimetry[b]	Epidemiology[b]	Statistics[b]	Support for LNT model[c]
LSS, Japanese atomic-bomb survivors (Grant et al., 2015)[d]	3	3	3	4
INWORKS (French, UK, US combined cohorts) (Richardson et al., 2015)	3	3	3	4
TB fluoroscopic examinations and breast cancer (Little and Boice, 2009)	3	3	2	4
Childhood Japan atomic-bomb exposure (Preston et al., 2008)*	3	3	3	4
Childhood thyroid cancer studies (Lubin et al., 2017; Ron et al., 1989)	3	3	3	4
Mayak nuclear workers (Sokolnikov et al., 2015)	2	2	3	3
Chernobyl fallout, Ukraine and Belarus thyroid cancer (Brenner et al., 2011; Zablotska et al., 2011)	3	2	2	3
Breast cancer studies, after childhood exposure (Eidemuller et al., 2015)	2	3	3	3
In utero exposures, Japan atomic bomb (Preston et al., 2008)	2	3	3	3
Techa River, nearby residents (Schonfeld et al., 2013)	2	2	2	2.5
In utero exposures, medical (Wakeford, 2008)	1	2	2	2.5
Japanese nuclear workers (Akiba and Mizuno, 2012)	2.5	2	3	2
Chernobyl cleanup workers, Russia (Kashcheev et al., 2015)	1	1.5	2	2
US radiologic technologists (Liu et al., 2014; Preston et al., 2016)[e]	1	2	2	2
Mound nuclear workers (Boice et al., 2011)	2	1.5	1.5	2
Rocketdyne nuclear workers (Boice et al., 2011)	2	2	2	2
French uranium processing workers (Zhivin et al., 2016)	2.5	3	1.5	2
Medical X-ray workers, China (Sun et al., 2016)	1.5	1.5	2	2[f]
Taiwan radiocontaminated buildings, residents (Hsieh et al., 2017)	2	1.5	1.5	2[f]
Background radiation levels and childhood leukemia (Kendall et al., 2013)	1.5	2	2	2
In utero exposures, Mayak and Echa (Akleyev et al., 2016)	1	1.5	2	1
Hanford ^{131}I fallout study (Davis et al., 2004)	2	3	1.5	1
Kerala, India, HBRA (Nair et al., 2007)	2	2	1.5	1
Canadian nuclear workers (Zablotska et al., 2014)	2.5	3	3	1
US atomic veterans (Caldwell et al., 2016)	3	3	3	1
Yangjiang, China, HBRA (Tao et al., 2012)	1.5	1	1	1[f]
CT examinations of young persons (Pearce et al., 2012)	1	1.5	1.5	1[f]
Childhood medical X-rays and leukemia studies (aggregate of >10 studies) (Little, 1999; Wakeford, 2008)	1	2	1.5	1[f]
Nuclear weapons test fallout studies (aggregate of eight studies) (Lyon et al., 2006)[g]	1.5	1	1.5	1[f]

[a] *A representative recent publication is listed for each study. Others are found in the text.*
[b] *Judged quality of the dosimetry, epidemiology and statistics scores: 1 = weak-to-moderate, 2 = moderate, 2.5 = moderate-strong, 3 = strong.*
[c] *Ratings of the support for LNT: 1 = essentially no support (null or negative; or unreliable and inconclusive), 2 = weak-to-moderate support, 3 = moderate support, 4 = strong support. Study ratings were based on reported solid cancer (or close surrogates) risk unless noted otherwise.*
[d] *Studies excluded: ecological studies of risks around nuclear sites (no dosimetry and extremely low exposures); the 15-Country worker study and other studies that overlap with the more recent INWORKS; the MPS is not yet completed, but published components of it are included separately; studies of genetic effects (since no human heritable risks have been shown); studies of tissue reactions (because these are generally not believed to be LNT).*
[e] *The dosimetry used in the Preston et al. (2016) study of breast cancer, based on the Simon et al. (2014) dosimetry, is significantly improved and would be rated "2" compared to the dosimetry that was used in other published epidemiologic analyses of this cohort. However, since little other epidemiology has been published using the new dosimetry, for the purposes of this Commentary, these studies are limited in their support for LNT.*
[f] *Considered "weak" support or "inconclusive" primarily because of weaknesses in the epidemiology, dosimetry or statistical risk modeling. The other studies scored as 1 (no support) had reasonable methodologies but provided little or no support for the LNT model because their risk coefficients were essentially zero or negative.*
[g] *Fallout studies were included as a group (they mostly had little or poor dosimetry and many were studies of aggregates rather than individuals, however, the Hanford ^{131}I fallout study was of better quality, so was identified separately).*

given in aggregate to each data set with regard to support of the LNT model. The NCRP report concludes "that no alternative dose–response relationship appears more pragmatic or prudent for radiation protection purposes than the LNT model." I fully concur with this scientific assessment and the resulting policy choice.

In my opinion, much of the LNT debate results from a failure on the part of some scientists, policy makers, and the public at large to understand how standards and regulations are developed. Scientists may overreach and argue science, especially scientific information they have personally developed, is crucial in the standard setting process. They argue for science-based standards. In some cases they may even argue that the scientific information translates into specific numerical standards different than current standards. I also argue for science-based standards and regulations, but reach a different conclusion.

In my opinion, it should be recognized that science informs policy judgments or decisions that are at the core of setting standards. Very few standards for any hazardous agents are actually set based on the use of specific data sets to arrive at specific risk target doses, that is, less than one extra cancer over a lifetime for a population of 10,000 individuals. Rather, numerical radiation protection standards intended to limit occupational or environmental exposures were initially set based on scientific judgment. The methods used have evolved over time. Many early standards were set by practitioners in a field. More recently, in the United States and many other countries, legislation has assigned standard setting responsibility to government agencies. At the same time, the process has increasingly called for greater involvement of the public. This approach places a premium on scientists learning to communicate more effectively with the public on matters that are at the interface of science and public policy. The legislative framework used in the United States for setting standards assigns the ultimate responsibility to a responsible government administrator, for example, the Administrator of the US Environmental Protection Agency or the Nuclear Regulatory Commission. I have previously reviewed a similar situation for the setting of national ambient air quality standards (McClellan, 1999).

43.18 Current radiation protection guidance

The recommended dose limits of the ICRP and NCRP at the time this chapter was written are shown in Table 43.16. Both the ICRP and NCRP systems make use of weighting factors to take account of the relative biological effectiveness of different types of radiation (Table 43.17). They also use tissue-weighting factors (Table 43.18).

TABLE 43.16 Recommended dose limits for ionizing radiation (mSv in a year).

Type of limit	ICRP	NCRP
A. *Occupational exposure* Stochastic effects Effective dose limit (cumulative)	20 mSv/yr averaged over 5 years, not to exceed 50 mSv in any one year	10 mSv × age
Annual	50 mSv/yr	50 mSvr/yr
Deterministic effects Dose equivalent limits for tissues and organs (annual) Lens of eye	150 mSv/yr	150 mSv/yr
Skin, hands, and feet	500 mSv/yr	500 mSv/yr
B. *Embryo/fetus exposure* Effective dose limit after pregnancy is declared	0.5 mSv/month	Total of 1 mSv to abdomen surface
C. *Public exposure (annual)* Effective dose limit, continuous or frequent exposure	No distinction between frequent and infrequent—1 mSv/yr	1 mSv/yr
Effective dose limits, infrequent exposure		5 mSv/yr
Dose equivalent limits Lens of eye	15 mSv/yr	15 mSv/yr
Skin and extremities	50 mSv/yr	50 mSv/yr
D. *Negligible individual dose (annual)*	No statement	0.01 mSv/yr

Source: ICRP (International Commission on Radiological Protection). 2007. The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103 and NCRP (National Council on Radiation Protection and Measurements): Recommendations for Limits for Exposure to Ionizing Radiation, NCRP Report No. 116 (1993).

TABLE 43.17 Recommended radiation weighting factors (W_R).[a]

Radiation type	Radiation weighting factor (W_R)
Photons	1
Electrons and mons	1
Protons and charged pions	2
Alpha particles, fission fragments, heavy ions	20
Neutrons	A continuous function of energy

[a]W_R *are used to adjust the absorbed dose in an organ or tissue to derive the equivalent dose.*
Source: ICRP (International Commission on Radiological Protection). 2007. The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103.

TABLE 43.18 Recommended tissue weighting factors (W_T)[a].

Tissue	W_T	Sum of W_T values
Bone marrow (red), colon, lung, stomach, breast, remainder tissues[b]	0.12	0.72
Gonads	0.08	0.08
Bladder, esophagus, liver, thyroid	0.04	0.16
Bone surface, brain, salivary glands, skin	0.01	0.04
Total		1.00

[a]*Used to derive the effective dose.*
[b]*Remainder tissues: adrenals, extrathoracic (ET) region, gall bladder, heart, kidneys, lymphatic nodes, muscle, oral mucosa, pancreas, prostate (♂), small intestine, spleen, thymus, uterus/cervix (♀).*
Source: ICRP (International Commission on Radiological Protection). 2007. The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103.

Both the NCRP and ICRP systems use a dose and dose rate effectiveness factor (DDREF) as an intermediate step to estimating total detriment from cancer and heritable stochastic effects of radiation. The ICRP recommends a DDREF of 2.0. The National Academies Biological Effects of Ionizing Radiation (BEIR) Committee has recommended a DDREF of 1.5 (BEIR VII, 2007). The DDREF as currently used combines both dose and dose rate. In my opinion, DDREF should be separated into two separate factors: a dose effectiveness factor (DEF) and a dose rate effectiveness factor (DREF). In my opinion, the information reviewed earlier would support a DREF of about 4.0. Brooks et al. (2009) have stated a similar view for internally deposited radionuclide exposure.

The ICRP framework for dose constraints reference levels is shown in Table 43.19 (ICRP, 2007; Wrixon, 2008). The detriment-adjusted nominal risk coefficients used for stochastic effects after exposure to radiation at low dose rates are shown in Table 43.20 (NCRP, 2007; Wrixon, 2008).

Both the ICRP and NCRP operate with an overarching goal to achieve radiation doses "as low as reasonably achievable." The standards are not intended to provide a license to allow exposure circumstances and radiation doses at the level of the standard.

Before concluding the consideration of radiation protection guidance, let me emphasize that this guidance has been developed to protect both workers and the public by controlling potential radiation exposures. This guidance also provides a useful context when considering and dealing with accidental releases such as a nuclear reactor or nuclear fuel reprocessing accident. However, an abundance of "common sense" needs to be exercised in dealing with accidents. For example, overzealous actions including evacuation of large populations to limit radiation exposures may result in greater loss of life from other causes such as traffic accidents than the estimated lives saved if the focus is inappropriately limited to radiation effects. In addition, the psychological stress associated with evacuations should not be discounted.

TABLE 43.19 Framework for dose constraints and reference levels.

Bands of effective dose, mSv (acute or annual)	Characteristics	Requirements	Examples
20 – 100	Controlled by action on exposure pathway	Consider reducing doses	Reference level for radiological emergency
1 – 20	Controlled by action or source or exposure pathway	For planned exposure situations, individual dose assessment and training	Constraints for occupational exposure. Constraints for comforters and carers of patients treated with radiopharmaceuticals. Reference level for radon in dwellings
< 1	Controlled by action on source	Periodic checks on exposure pathways	Constraints for public exposures in planned situations

Source: ICRP (International Commission on Radiological Protection). 2007. The 2007 Recommendations of the International Commission on Radiological Protection, ICRP Publication 103.

TABLE 43.20 Detriment-adjusted nominal risk coefficients for stochastic effects after exposure to radiation at low dose rate (10^{-2} per Sv).

	Cancer		Heritable effects		Cancer total detriment	
Exposed population	1990[a]	2007[b]	1990	2007	1990	2007
Whole	6.0	5.5	1.3	0.2	7.3	5.7
Adult	4.8	4.1	0.8	0.1	5.6	4.2

[a] *1990 cancer values based upon fatal cancer risk weighted for non-fatal cancer, relative life years lost for fatal cancers and life impairment for nonfatal cancer.*
[b] *2007 cancer values based upon data on cancer incidence weighted for lethality and life impairment.*

43.19 Summary

It is my opinion that our collective knowledge of the health effects of exposure to ionizing radiation from external sources or internally deposited materials exceeds that existing for any other single potentially hazardous agent or class of agents. Further, it is my professional opinion that the high level of knowledge of radiation effects today strongly supports current radiation protection standards. Despite that high level of knowledge, there are few potential hazards that evoke more fear and concern than potential radiation exposures. I submit that in some situations the level of fear and concern is irrational and severely inhibits public discourse on anything to do with radiation or nuclear activities. This includes consideration of the use of nuclear reactors versus fossil-fueled power plants or solar or wind to generate electrical power.

The health effects of explosion of a nuclear weapon are directly evident from the bombing of Hiroshima and Nagasaki, Japan, with weapons that had yields on the order of 20 kt. The effects were devastating, with hundreds of thousands of people killed from the blast overpressure, thermal radiation, and direct ionizing radiation. A cohort of survivors enrolled in 1950 is still being followed with the primary effect observed being a small increase in radiation attributable to cancer deaths over and above the substantial background incidence of cancer. Nuclear weapons are now in the hands of about a dozen countries and other nations are attempting to develop nuclear weapons. On the order of several thousand weapons have been tested, with yields extending up to the megaton range. Political efforts to control the spread of nuclear weapons have been a dominant international political issue for the past three-quarters of a century and must be continued!

The threat of terrorists using nuclear weapons is a continuing concern. A more realistic concern is for rogue nations to use nuclear weapons in the face of uncertainty

as to how other nations will respond. There will be no winners in a war that involves nuclear weapons! Concerns continue for terrorists attempting to create and use weapons that disperse radioactivity. The threats of such usage continue to generate fear. In my opinion, the potential for such devices to injure substantial numbers of individuals is quite low. However, the efforts to clean up contamination from use of any radioactivity-dispersing devices could be substantial and would certainly create additional fear and confusion. Terrorism of any kind must be curbed!

43.20 Personal perspective

My life and professional career have been profoundly impacted by the events I have just discussed. In 1943, my parents joined the work force that would ultimately total about 50,000 workers constructing the Hanford, WA complex. At the time they were recruited, it was known as the "Hanford Engineering Works." It would ultimately become the Hanford Atomic Products Operation. In the summer of 1944, I joined my parents living in Richland, WA, just south of the Hanford complex. I was underage for radiation work, I started the third grade. Coincidently, Cantril and Parker (1945) who would become my mentors were writing a radiation protection plan for Hanford. Growing up in a very technocratic community I was influenced by many engineers and scientists employed at Hanford who were my neighbors. As a high school student I participated in experiments on ^{131}I effects in sheep. Later as a university student I spent three summers as an intern at Hanford conducting research on ^{65}Zn, ^{131}I, ^{239}Pu, and other radionuclides. After receiving my Doctor of Veterinary Medicine degree from Washington State University in 1960, I joined the staff of the Hanford Laboratories, which were headed by Herbert M. Parker and conducted research on numerous radionuclides in laboratory and domestic animals working with Leo K. Bustad. Phillip Abelson would also become a mentor.

I spent 1965–66 in a term assignment with the US Atomic Energy Commission's (AEC) Division of Biology and Medicine. I was fortunate to have Paul S. Henshaw, the author of the 1941 classic radiation effects paper, as my office mate. Glenn Seaborg was at the helm of the AEC as Chair of the five-person Commission. He was influential in encouraging me to accept a position in September 1966 with the Lovelace Medical Center in Albuquerque, NM, providing leadership for a new research program. That program focused on developing an improved scientific basis for understanding the potential health effects of a catastrophic nuclear reactor accident such as ultimately occurred on April 26, 1986, in Chernobyl, Ukraine.

For over four decades the Lovelace research program was a major contributor to the world's literature on the health effects of inhaled fission product radionuclides and plutonium. In the mid-1970s, the Lovelace program diversified and used its substantial expertise in aerosol science and inhalation toxicology to address the health consequences of airborne emissions from hydrocarbon-fueled energy technologies such as diesel engines. Later, starting in 1988, I provided leadership for the Chemical Industry Institute of Toxicology (CIIT) in Research Triangle Park, NC, a research laboratory focused on the mechanisms of actions of chemical agents. While at CIIT and continuing to the present, I have maintained a keen interest in understanding the human health risks of radiation exposure.

43.21 Dedication

This chapter is dedicated to my parents, my teachers, and fellow students in the Richland, WA, schools and the many colleagues who I have had the pleasure of working with for over 60 years in the radiation field. In 1943, my parents made a bold move from Minnesota to the state of Washington to become a part of the Hanford Engineering Works, which would later become known as the Hanford Atomic Products Operation. They did not know what products were to be produced nor could they have possibly envisioned the impact the key product, plutonium, would have on the world. They viewed the association with the Hanford site as an adventure and opportunity. They had interesting jobs that had impact. My father was a chemical process operator. As a youngster, I knew he must do some unusual kind of work because periodically there was a gray box on our front porch containing bottles to collect his urine—my introduction to bioassay systems. Later, I learned he worked in the plant that separated ^{239}Pu from irradiated fuel. My mother was a secretary to the principal in multiple schools. Later, I would appreciate that this position is critical to the success of any organization; those individuals are de facto chief operating officers.

My siblings and I were fortunate that the Richland schools had exceptional teachers and demanding curricula. We had fellow students who were quite accomplished. They and their parents came from all over the United States. In high school, our sports teams after 1945 were known as the "Bombers" and there was a large mural of a Bomber on the side of the high school. Our logo was a mushroom cloud. In 1954, I graduated from Richland Columbia High School. Not surprising, the graduates followed a multitude of career paths. Some followed in the footsteps of their parents and immediately went to work on the Hanford site. Others joined the military. Many went to college and, some like myself, followed career paths related to nuclear activities.

One of my classmates, David Koeppen, had a little different background. His father had a ranch close to Richland. I had the good fortune to work on the Koeppen Ranch in 1953 and learned the value of hard physical

work. David and I were good friends. In the fall of 1954, David joined the US Navy and quickly rose to the rank of Chief Petty Officer. He traveled around the world under the sea on nuclear submarines. As I was finishing this book chapter, I attended the 65th reunion of the Columbia High School Class of 1954. I had a wonderful visit with David as we connected the dots on what we had both done during the last 65 years. During that visit I learned that while I was busy studying the health effects of radiation, David had a remarkable career that involved up close contact with nuclear activities. Most of his 20 years in the US Navy were spent on nuclear-powered submarines. He was frequently berthed and worked close to a nuclear reactor! Moreover, the submarines he crewed were armed with ballistic missiles topped with nuclear war heads. During our visit I learned that one of the highlights of his career occurred when he served on the nuclear-powered submarine, the USS Ethan Allen, from October 1, 1960, to October 18, 1968. He was on board the submarine on May 6, 1962, south of Hawaii when it participated in Operation Dominic—Frigate Bird. It fired a Polaris ballistic missile with a nuclear war head that traveled 1200 miles in 12.5 min and on reentry at about 11,000 ft detonated near Johnston Island in the South Pacific. David noted with justifiable pride: "We put that war head right in the old pickle barrel as planned." Ironically, in the 1980s, I would serve on an advisory committee guiding cleanup of the Pacific islands impacted by nuclear weapons testing. We were two classmates whose paths were different, yet so close at times! I am saddened to note that after the reunion David flew his private plane toward his home in southern Idaho, crashed in the mountains of Oregon, and was killed. I salute his distinguished service to our nation and will forever treasure our friendship.

In closing this dedication, I wish to also note my appreciation to the many colleagues that I worked with over the decades to expand the world's knowledge of the health effects of radiation and radionuclides. Our research had two hallmarks. It was purpose driven, directed at addressing uncertainties in our scientific ability to address human risks of exposure to radionuclides. It was research that could not have been conducted without teamwork from individuals that had complementary talents and were willing to work together to achieve important goals. It was a pleasure to be a part of that team and learn from and be mentored by many of my team mates.

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Chapter 44

Clinical and cellular aspects of traumatic brain injury

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44.1 Introduction

Traumatic brain injury (TBI) is a sporadic mechanical impact to the head that leads to acute or chronic impairments in neurological function. It is the most common cause of death in North America in individuals between ages 1 and 45 years (Rutland-Brown et al., 2006; Rosenfeld et al., 2012), accounting for 30.5% of all injury-related deaths. There are approximately 1.7 million cases of TBI annually in the United States, leading to 1.4 million emergency department (ED) visits, 275,000 hospitalizations, and 52,000 deaths. Costs of TBI, including direct medical expenses and indirect costs related to loss of productivity, amount to an estimated $76.5 billion each year.

TBI occurs most commonly to children aged 0−4 years, adolescents aged 15−19 years, and adults older than 65 years. Unfortunately, the prevalence of TBIs has increased in recent years, rising by 58% (from 520 to 820 per 100,000) between 2001 and 2010. In that same period, the number of TBI-related deaths decreased by 8%, likely due to better understanding of how to manage TBIs. The injury mechanisms of TBI-related deaths varies between age groups (cdc.gov, 2019). In persons 0−4 years old, TBI-related deaths often occur as a result of child abuse. In young children to young adults, TBI-related deaths are usually caused by motor vehicle accidents. In middle to late adulthood, self-inflicted TBIs are the major cause of death. In the elderly, falls account for the majority of deaths by TBI. TBI-related death rates are highest in adults older than 75 years, and males are more likely to die from TBI than females across all age groups (Faul et al., 2010).

This chapter summarizes our understanding of the cellular and molecular mechanisms that account for neurological abnormalities observed after TBI. The points of this work can be summarized as follows:

- TBI includes both primary and secondary injuries.
- Immediate care after TBI shares the same principles as advanced life support.
- A team of healthcare specialists work with the patient and family to facilitate recovery post-TBI.
- Primary injuries lead to necrosis and apoptosis.
- Secondary injuries lead to apoptosis or alterations in synaptic function.
- Several pathological changes suggest that TBI is a risk factor for other neurological diseases.

44.2 Traumatic brain injury mouse models

Our understanding of the cellular and molecular changes that occur after TBI have been advanced through the use of animal models of TBI (Fig. 44.1). In general, animal models of TBI deliver mechanical forces to the brain or skull in a controlled manner in order to replicate known neuropathology, rather than the physical parameters of injury. Accordingly, many of the outcomes in animal models mimic those observed in human TBI, such as concussion, contusion/hemorrhage, and *diffuse axonal injury (DAI)*. As with any experimental model, however, there is a trade-off between reproducibility and physiological relevance. A brief description of commonly used animal models of TBI follows. Readers interested in further details about different animal models of TBI should read the work by Xiong et al. (2013).

Highly reproducible models include fluid percussion injury and controlled cortical impact. In *fluid percussion injury*, a pressure pulse is sent through a fluid reservoir

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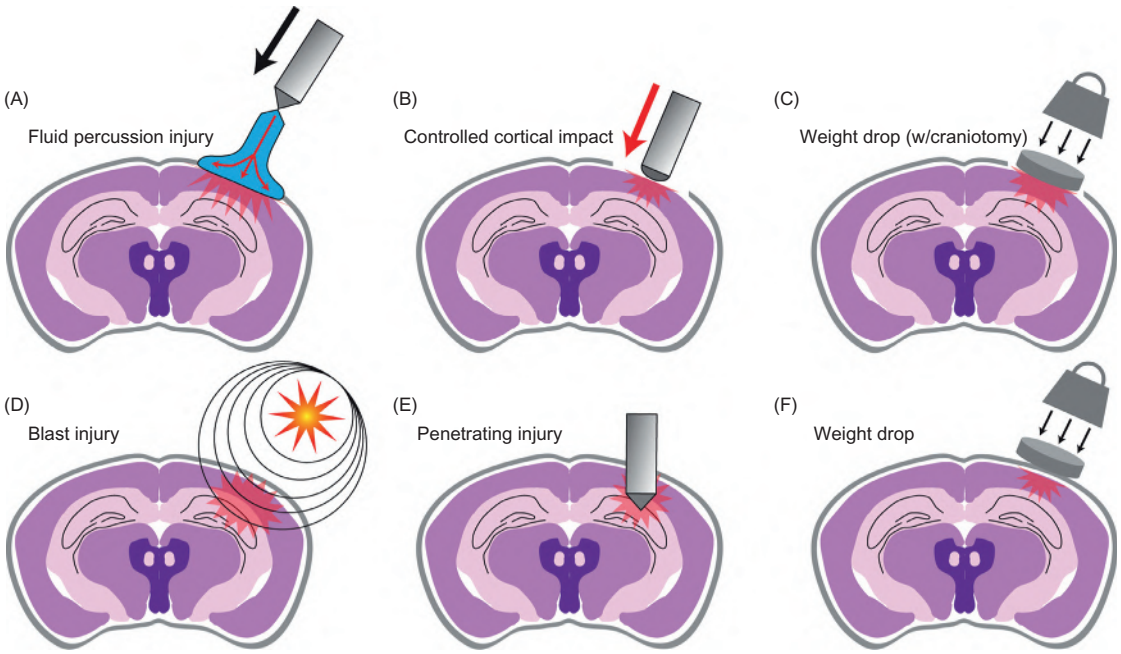

FIGURE 44.1 Mouse models of TBI. (A) Fluid percussion injury uses a saline-filled reservoir resting on the dura to reliably transfer energy from a piston onto the brain. (B) Controlled cortical impact uses a pneumatic piston or electromagnetic actuator to drive a rod directly onto the skull or dura. (C & F) Weight drop models apply force by dropping weights from set heights. Compared to fluid percussion injury and controlled cortical impact, weight drop models are relatively inexpensive to implement but produce more variable injuries. (D) Blast injuries use pressurized air waves to delivery energy to brain tissue. Due to the spread of blast waves, peripheral tissues are also prone to damage. (E) Penetrating injuries drive small probes into the brain parenchyma. These models uniquely simulate the effects of penetrating injuries, while all other models simulate closed head injuries.

placed on the exposed dura. This pressure pulse displaces the brain to generate focal and diffuse injuries. *Controlled cortical impact* drives a rigid object directly onto the exposed dura. Both of these are open models of TBI, requiring removal of a portion of the skull (craniotomy). This decreases variability of the injury but reduces the physiological relevance to closed TBI.

More biomechanically relevant models include blast injuries, penetration injuries, weight-drop, and repeated mild injuries. *Blast injuries* deliver a pressurized gas wave to the head. Because this model uses a pressure wave that is difficult to focus, the animal's body must be protected using a Kevlar jacket. Blast injuries are excellent models of TBIs suffered as a result of improvised explosive devices, which are the primary cause of head injuries in modern military conflicts (see Chapter 45: Neurological effects and mechanisms of blast overpressure injury). *Penetrating injuries* drive a projectile into the brain with high energy to mimic TBIs that occur from gunshot wounds. In *weight-drop models*, a free-falling weight is dropped on the exposed skull. In some weight-drop models, a craniotomy is performed to increase reproducibility.

Repeated mild injury models are of particular interest because they mimic injuries that occur in contact sports. In addition, mild TBIs account for between 70% and 90% of all TBIs, although this figure is likely to be an underestimate due to the difficulty in diagnosing mild injuries. Repeated injuries have been delivered using fluid percussion, blast, and weight-drop models. Although more characterization is needed, repeated mild injury models suggest that even mild TBIs can produce rapid, disastrous effects (Fehily and Fitzgerald, 2017). Areas with repeat injuries show exacerbated neuropathology, including reactive gliosis, myelin degeneration, axonal damage, tau phosphorylation, blood–brain barrier (BBB) permeabilization, and reduced cerebral blood flow. Repeated mild injuries also worsen behavioral symptoms, such as learning impairments and behaviors suggestive of anxiety and depression. These findings are consistent with data from athletes and active military personnel showing that repeated head injuries worsens the severity of neurological symptoms.

44.3 Clinical manifestations and management of traumatic brain injury

44.3.1 Classifying traumatic brain injury using the Glasgow Coma Scale

TBI is a heterogeneous entity. The severity of TBI can be classified according to various injury scoring systems, with each carrying different impacts on prognosis and treatment. The most commonly used scoring system is the

TABLE 44.1 Using the GCS to assess head injury severity.

Behavior	Response	Score
Eye opening	Open and blinking spontaneously	4
	Open to verbal stimuli	3
	Open to painful stimuli	2
	No response	1
Verbal	Aware of self, time, and location	5
	Slight confusion	4
	Inappropriate replies	3
	Incomprehensible replies	2
	No reply	1
Motor	Follows commands	6
	Appropriate purposeful withdrawal from pain	5
	Normal flexion in response to pain	4
	Abnormal flexion in response to pain	3
	Extension in response to pain	2
	No response	1

Classification: Coma: 3. Severe head injury: 4–8. Moderate head injury: 9–12. Mild head injury: 13–15.

Glasgow Coma Scale (GCS) at initial presentation (Vos et al., 2002; Moppett, 2007) (Table 44.1). A GCS score of 13–15 is considered mild injury, 9–12 is moderate, and 8 or less represents severe TBI. The initial severity of TBI has prognostic value regarding the long-term outcome of the injury (Thornhill et al., 2000). Although the majority of TBI cases are mild (>75%) (Faul et al., 2010), the minority of patients presenting with moderate or severe TBI have a very poor prognosis, with mortality rates as high as 55%. Of all patients with a post-TBI GCS of 12 or less, 2% die within the first hour and 8% die within the first 6 h (Peek-Asa et al., 2001).

Although the GCS excels as a universal tool for TBI classification because of its simplicity, reproducibility, and prognostic power, its use may sometimes be limited by clinical confounders, such as anesthesia, sedation, paralysis, and coexisting intoxication. These confounding factors are especially prominent in patients with a low GCS score (Stocchetti et al., 2004). Hence, CT-based scoring systems are sometimes used by neurotrauma centers for a more objective classification on TBI. These include the Marshall scale, which has been shown to be accurate in predicting risks of increased *intracranial pressure (ICP)* but lacks reproducibility in cases of multiple brain injuries (Marshall et al., 1992), and the Rotterdam scale, which was developed more recently to overcome the limitations of the Marshall scale but still lacks validation in large populations (Maas et al., 2005).

44.3.2 Coma recovery scale to track meaningful changes with severe traumatic brain injury

Severe TBI is often described as a disorder of consciousness. After a severe injury, there can be a disruption of awareness of one's self and the surrounding environment, the inability to interact with others, and disorders of consciousness. Disorders of consciousness include coma, vegetative state, and minimally conscious state. Disorders of consciousness can be assessed using the *Coma Recovery Scale-revised* (*CRS-R*, Table 44.2). The CRS-R consists of 23 items with six subscales of sensory-evoked behaviors that are arranged in a hierarchy from absent to reflexive behaviors to cognitive mediated behaviors (Giacino et al., 2004). A maximum score of 23 indicates cognitive mediated behaviors and normal consciousness, whereas lower scores indicate absent of reflexive behaviors and a disturbance in consciousness (Giacino et al., 2004). The CRS-R can be used to assist with differential diagnosis, prognostic assessment, and treatment planning in individuals with disorders of consciousness.

TABLE 44.2 Using the CRS-R to track meaningful changes with severe TBI.

Behavior	Response	Score
Auditory	Consistent movement to command	4
	Reproducible movement to command	3
	Localization to sound	2
	Auditory startle	1
	None	0
Visual	Object recognition	5
	Object localization: reaching	4
	Visual pursuit	3
	Fixation	2
	Visual startle	1
	None	0
Motor	Functional object use	6
	Automatic motor response	5
	Object manipulation	4
	Localization to noxious stimulation	3
	Flexion withdrawal	2
	Abnormal posturing	1
	None/flaccid	0
Oromotor	Intelligible verbalization	3
	Vocalization/oral movement	2
	Oral reflexive movement	1
	None	0
Communication	Functional: accurate	2
	Nonfunctional: intentional	1
	None	0
Arousal	Attention	3
	Eye opening without stimulation	2
	Eye opening with stimulation	1
	Unarousable	0

44.3.3 Intracranial pressure

TBI is often associated with brain edema, which increases ICP. It is important to note that there are two types of brain edema: cytotoxic brain edema and vasogenic brain edema. Cytotoxic brain edema is caused by disturbances in the osmotic balance of neural cells, such that increased water influx leads to necrosis, as observed in cases of excitotoxicity. Vasogenic brain edema involves disruption of BBB integrity. The BBB is a series of tight junctions between vascular epithelial cells. These tight junctions are dynamic structures that are maintained by the astrocytic endfoot processes that line the vascular epithelium. In pathological states (such as TBI), the tight junctions between epithelial cells are replaced by porous structures that allow the entry of large macromolecules (and their accompanying solvent) into the brain.

To understand the relationship between ICP and brain herniation, it is important to conceptualize the cranium as a rigid vault packed with three major components: brain

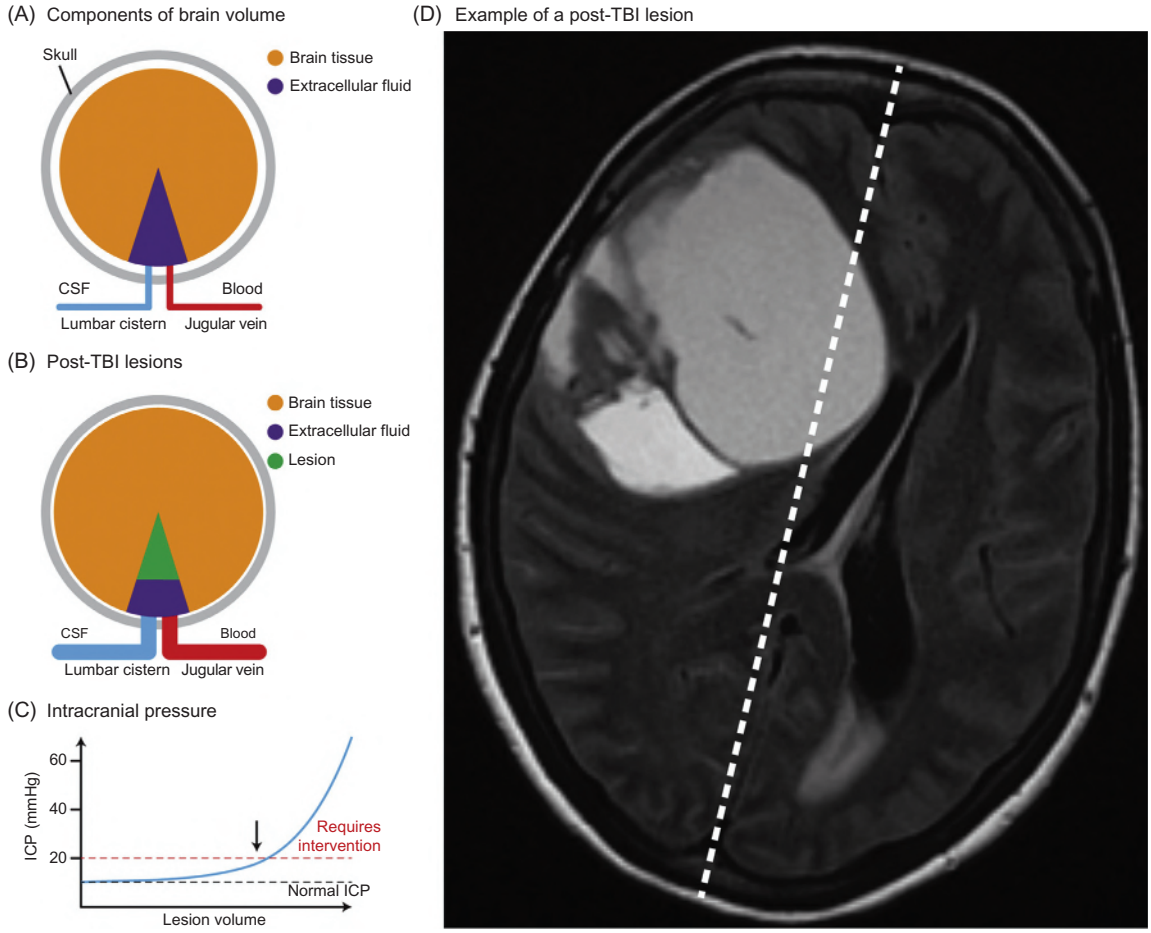

FIGURE 44.2 Regulation of brain volume is critical for maintaining safe ICP. (A) Brain tissue and the extracellular fluid (comprising blood and CSF) contribute to normal brain volume. (B) Brain lesions that occur after TBI increase intracranial volume. (C) An initial increase in intracranial volume only leads to a minimal increase in ICP because of a compensatory outward flow of CSF and blood. Once this protective mechanism has been exhausted (arrow), ICP begins to increase with a steep slope. (D) Magnetic resonance imaging (MRI) showing midline shift of brain contents secondary to a decompensated increase in intracranial volume (in this case by a growing tumor). Loss of sulci/gyri prominence and asymmetric compression of ventricles are seen. White line depicts the expected midline of the brain.

tissue (~80%), blood (~8%), and CSF (~12%). The physical law of compliance governs the relationship between ICP and the volume of these three components: an increase in pressure leads to a decrease in volume. Blood and CSF, both being fluids, are much more easily displaced than the solid brain parenchyma. Hence, when intracranial volume increases (e.g., due to acute intracranial bleeding), CSF and blood are the first components to be "pushed out" of the cranial vault. CSF drains from the cranium into the communicating subarachnoid space in the spinal column, whereas venous blood drains back to the heart. The flexibility of CSF and blood volume within the cranium creates a compensatory "cushion," such that increases in intracranial volume do not lead to elevated ICP initially. This "cushioning" effect lasts for as long as there is blood or CSF to be displaced. However, when intracranial volume expands beyond a certain level, this compensatory mechanism becomes exhausted. ICP will now begin to increase steeply with further volume expansion (Fig. 44.2), and brain tissue now becomes the only other element left to be compressed by the increased ICP. Such compressive forces can lead to herniation of the brain across various weak points in the brain or across the tentorium. Brain herniation manifests with a wide variety of clinical signs and symptoms, depending on the area of the brain being compressed. The most dreaded complication is brain stem compression, which leads to suppression of many neurological functions important for survival, such as regulation of respiration and circulation. Other ominous signs suggestive of impending herniation include unilaterally or bilaterally fixed and dilated pupil(s), decorticate or decerebrate posturing, and the Cushing triad (bradycardia, hypertension, and respiratory depression).

Because a TBI that increases ICP is likely to have a poor outcome, it was thought that preventing edema

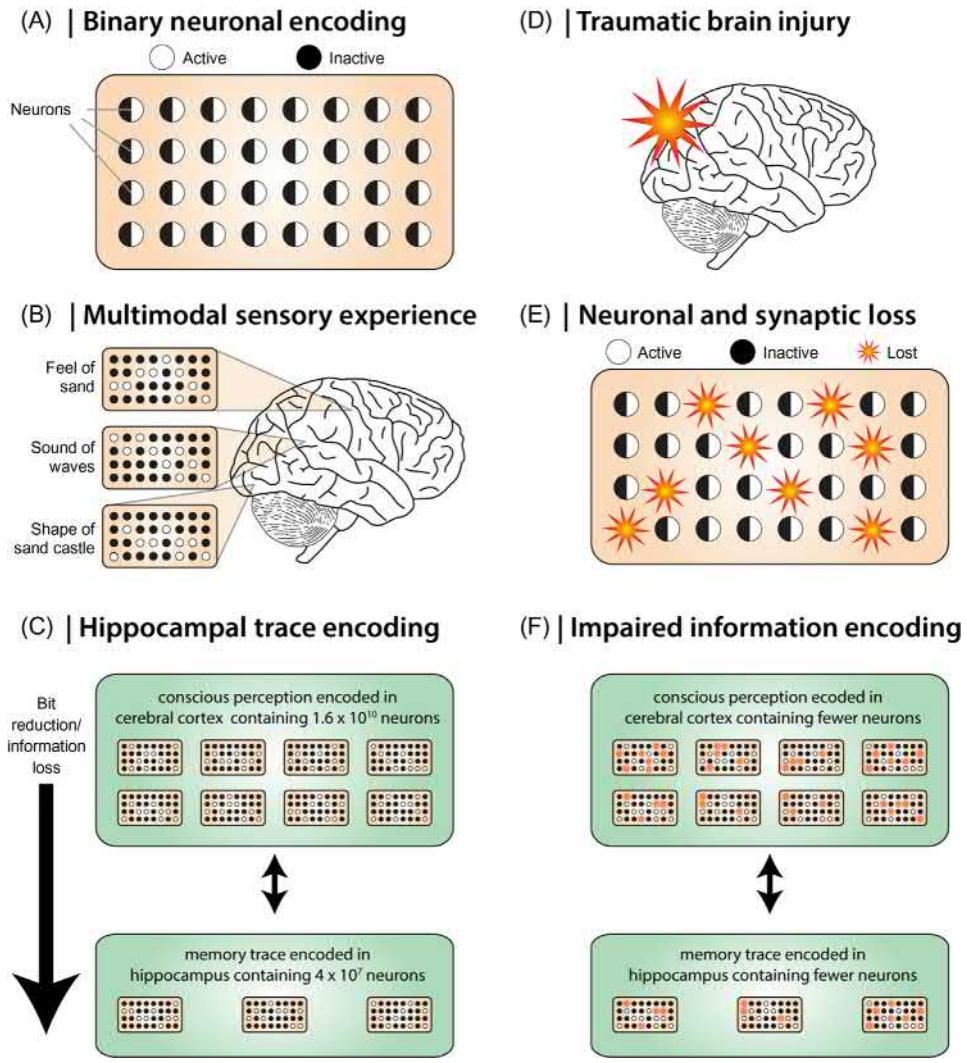

FIGURE 44.3 Cortical and hippocampal networks integrate sensory information. (A) The brain is often thought of as a computer. The binary code used by the brain is the activity or inactivity of specific neurons. Even a simple network of 30 neurons can encode more than 100 million (2^{30}) different information states. (B) Different patterns of neuronal activity in distinct brain regions encode a variety of sensory information. (C) Extensive interconnections between the cortex and hippocampus via the entorhinal cortex allow the encoding of memory traces. Because there are fewer neurons and synapses in the hippocampus than in the cortex, information is lost during the encoding of memory traces, which may explain why memories are not as vivid as primary sensory experience. (D, E) The loss of neurons that occurs after a TBI reduces the amount of information we can encode. In fact, DAI may also sever connections between brain regions. (F) The loss of neurons and synapses impairs information encoding, storage, and retrieval, causing memory, cognitive, sensory, and motor deficits.

would have therapeutic benefits. However, antiedematous therapy (e.g., steroids, superoxide dismutase, calcium antagonists, and inhibitors of bradykinin and glutamate receptors) has not proved effective in clinical trials conducted after TBI (Unterberg et al., 2004). This suggests that management of functional impairments associated with TBI will require targeting more upstream changes in cell signaling pathways. In line with this, intracranial lesions do not always lead to increased ICP, and increased ICP does not always equate with increased risk of herniation. In certain conditions, such as pseudotumor cerebri, ICP can be chronically elevated but well-compensated with decreased ventricular size and intracranial CSF volume. Hence, patients with pseudotumor cerebri are not at significantly increased risk for brain herniation. Neuroimaging can be helpful in assessing the degree of intracranial distortion from elevated ICP. Concerning radiological findings suggestive of decompensated ICP elevation include loss of gray/white distinction, loss of sulci/gyri prominence, asymmetrical compression or enlargement of ventricular space, mass effect (distortion of size or position of normal brain structures), and midline shift (Fig. 44.3). These findings should prompt immediate medical or surgical therapy.

44.3.4 Primary and secondary brain injury

TBI is often divided into two distinct but related phases: primary brain injury and secondary injury (Werner and Engelhard, 2007; Greve and Zink, 2009). Clinically, primary brain injury often requires surgical attention, whereas secondary injury is managed in the intensive care setting, where the prevention and treatment of these secondary insults become the major focus of neurotrauma intensivists.

Primary brain injury occurs at the time of the initial mechanical impact to the skull. The transfer of external mechanical forces to intracranial contents can lead to intra-axial damage and/or extra-axial injuries. *Intra-axial damage* involves the brain parenchyma. Focal cerebral contusions occur most commonly after TBI. Injuries involving abrupt acceleration/deceleration result in direct resonant impact of the brain with the base of the skull. Hence, the basal, frontal, and temporal areas are especially susceptible to contusion due to their anatomical distribution in relation to the bony areas of the cranial vault. In addition, shearing forces during mechanical impact can cause DAI. Patients with DAI often present with profound coma without elevated ICP, and often have a very poor prognosis. The cellular and molecular outcomes of intra-axial damages are described in greater details in other sections of this chapter.

Extra-axial damage involves non-brain cranial structures. Penetrating trauma, blast waves, or direct impact can result in skull fracture and rupture of intracranial vessels. Depending on the location of the impacting force, different types of hematomas may result.

1. *Epidural hematoma (EDH)*. Direct impact to the lateral surfaces of the head may cause rupture of the middle meningeal arteries embedded within the dura, forming an EDH. EDHs usually appear as a well-demarcated, biconvex lens-shaped density on CT scan. Bleeding in EDH is usually from a high-pressure arterial source, but is initially tamponaded by the tightly tethered dura. However, as the hematoma expands and strips the dura from the skull, it gradually creates an intense headache. Classically, patients with EDH are described as having a lucid period right after injury, when bleeding is still contained and tamponaded by the dura. This lucid period is followed by sudden loss of consciousness, when expansion of hematoma becomes large enough to cause a significant increase in ICP and compression or herniation of brain tissue.
2. *Subdural hematoma (SDH)*. Impact to the frontal regions, as well as shearing forces from linear or rotational acceleration/deceleration, can tear the subdural bridging veins and result in SDH. On CT, SDHs appear as poorly demarcated crescent-shaped densities. Unlike EDH, SDH occurs between the dura and the arachnoid layers. Hence, SDH lacks the additional layer of "protection" offered by the rigid dura. Clinically, patients with SDH present with gradually increasing confusion and headaches. Acute SDH has a high mortality rate and often requires prompt surgical evacuation.
3. *Subarachnoid hemorrhage (SAH)*. SAH refers to bleeding that occurs in the subarachnoid space, which is between the arachnoid and pia mater and is normally filled with cerebrospinal fluid (CSF). The majority ($>85\%$) of SAHs occur in patients with a preexisting intracerebral vascular lesion, most commonly an aneurysm (van Gijn and Rinkel, 2001), which serves as a functional weak point that is susceptible to rupture when intravascular pressure is elevated. However, in the setting of trauma, sufficient external forces could lead to rupture of intracerebral vessels without preexisting vascular abnormalities. Radiologic clues for traumatic SAH include localized bleeding in the superficial sulci, adjacent skull fracture, cerebral contusion, as well as external evidence of traumatic injury (Rinkel et al., 1993). Clinical suspicion for SAH should be high for patients reporting sudden, severe headaches (often described as "thunderclap" headache or "the worst headache of my life"). In these cases, emergent CT of the head should be obtained. If CT fails to identify intracranial bleeding but clinical suspicion for SAH remains high, then a lumbar puncture must be performed to look for blood in the CSF.

Intracranial bleeding after TBI can be further exacerbated by *coagulopathies*, which develop in up to one-third of TBI patients (Zehtabchi et al., 2008; Allard et al., 2009; Wafaisade et al., 2010). Coagulopathies may result either from underlying anticoagulation medications (e.g., warfarin, clopidogrel) or from TBI-induced platelet dysfunction, release of systemic tissue factor, and activation of endogenous anticoagulation pathways (Maegele, 2013).

Secondary injuries of TBI result from cellular perturbations caused by primary injury. Secondary injury can happen at any later time point after the initial impact. Although the primary injury of TBI is a major predictor of outcome, secondary insults can contribute to further worsening of prognosis (McHugh et al., 2007). The mechanisms of secondary injury include neurotransmitter-mediated excitotoxicity, free-radical injury to cell membranes, electrolyte imbalances, mitochondrial dysfunction, inflammatory responses, apoptosis, secondary ischemia from vasospasm, focal microvascular occlusion, and vascular injury (Werner and Engelhard, 2007). These events can lead to cerebral edema and further increases in ICP. The detailed molecular mechanisms of these events are described later in this chapter.

Individuals who have a severe brain injury, defined as a GCS <8, are at risk for developing a secondary complication called *paroxysmal sympathetic hyperactivity*, more commonly referred to as *brainstorming*. A brainstorming episode is thought to be an exaggerated stress response due to increased sympathetic nervous system activity. Symptoms can include posturing, dystonia, hypertension, tachycardia, pupillary dilation, diaphoresis, hyperthermia, and tachypnea (Kishner et al., 2006; Thorley et al., 2001;

Baguley et al., 2004). Immediate treatment of symptoms with mediations and environmental modifications must be administered to avoid the long-term effects of these episodes.

A critical aspect in post-TBI intensive care management is the avoidance of these secondary brain insults, which would otherwise be well tolerated but can exacerbate neuronal injury in cells made vulnerable by the initial TBI. Of particular importance is the prevention of hypotension and hypoxia (which decrease substrate delivery of oxygen and glucose to injured brain), fever and seizures (which may further increase metabolic demand), and hyperglycemia (which may exacerbate ongoing injury mechanisms), as discussed in the following sections.

TBI management focuses on stabilization of primary injury and prevention of secondary injury. Prolonged hospital stays due to increased risk for deep vein thrombosis, muscular contractures, spasticity, pressure sores, generalized muscle weakness, and overall impaired functional mobility must be addressed early on in medical care. TBI patients have limited opportunities for mobility in early recovery and are at risk for developing these difficult secondary complications. Below, we discuss management strategies during the early resuscitation period, postresuscitation intensive care therapeutic goals, and long-term rehabilitation. It is important to remember that at any period after TBI, the main goal is to avoid hypoxia and hypotension, the two most critical factors in predicting post-TBI outcome (Manley et al., 2001; Brain Trauma Foundation, 2007a; McHugh et al., 2007). The presence of prehospital hypoxia (PaO_2 <60 mmHg) and hypotension (systolic pressure <90 mmHg) is strongly associated with a poorer outcome (odds ratio $= 1.7-2.6$ and $2.1-3.4$, respectively) (McHugh et al., 2007).

44.3.5 Immediate care

For TBI patients and patients on advanced life support (ALS), the primary focus is on securing airway, breathing, and circulation (i.e., the ABCs of ALS). This may require intubation to maintain adequate oxygenation and fluid resuscitation or use of pressors to maintain perfusion pressure. The patient's heart rate, blood pressure, respiratory rate, and temperature should be continuously monitored. Neurological examination should be performed to assess the GCS score, and should be reassessed at regular intervals. A secondary survey should be performed to assess other extracranial injuries. All patients with TBI should be assumed to have an unstable cervical spine, and precautions should be applied to stabilize the spine (e.g., using a C-collar, "log-roll" precautions) to avoid cervical spinal cord or brain stem impingement during movements of the head. Blood should be sent to check for complete blood count, glucose, electrolytes, blood gases, and pH, as well as toxicology screen. As soon as the patient is stabilized, CT scan of the head should be obtained. CT can reveal skull fractures, intra-axial or extra-axial hemorrhages, significant brain contusions, and edema, which can guide downstream treatment strategies. If initial neurological examination or imaging shows findings suggestive of decompensated ICP elevation and impending herniation (Fig. 44.2), then emergent treatment should be started immediately, including head elevation, maneuvers to improve venous drainage, and osmotic therapy.

44.3.6 Surgical management

Timing and indications for surgical intervention after TBI are based on neurological status and radiological findings. Intracranial bleeding causing significant midline shift should be evacuated surgically regardless of hematoma size. In the absence of midline shift, surgical evacuation of hematomas is recommended if the blood volume is large, or if the patient has a GCS less than 8. For penetrating injuries and skull fractures, surgery for superficial debridement and repair of dural tears and CSF leaks are often recommended. However, debridement of deeper tissues and aggressive extraction of intracranial foreign bodies and bone fragments have not been shown to improve outcome or prevent delayed infection, as long as prophylactic broad-spectrum antibiotics are administered in the setting of penetrating injuries. Occasionally, decompressive craniectomy (removal of a substantial portion of the skull) is performed in conjunction with hematoma evacuation to help decrease ICP. However, the efficacy of decompressive craniectomy in outcome improvement is still controversial, and ongoing clinical trials are under way to determine the benefits versus risks of this technique (Jiang et al., 2005; Cooper et al., 2011; Ho et al., 2011; Servadei, 2011).

44.3.7 Targeted therapies to prevent secondary injury

After the initial resuscitation phase, TBI patients are monitored in the intensive care unit (ICU), where the major goal of care is to maintain cerebral and systemic perfusion, to correct electrolyte and coagulation abnormalities, and to minimize secondary insults.

44.4 Maintenance of adequate cerebral perfusion improves outcome after traumatic brain injury

Adequate *cerebral perfusion pressure (CPP)* is essential to prevent cerebral ischemia or toxic pooling of inflammatory mediators. Optimal CPP after TBI is between 50

and 70 mmHg, with 60 mmHg being the target (Elf et al., 2005; Jaeger et al., 2010). CPP is equal to mean arterial pressure (MAP) minus ICP (CPP = MAP − ICP). Because CPP cannot be readily measured, MAP and ICP are used as surrogate measures. Goal CPP should be achieved first by reducing ICP, then optimizing MAP.

Patients with TBI should be positioned strategically to minimize ICP elevation. The head of the bed should be elevated to 30°. The neck should be in a neutral position without external compression to ensure adequate venous drainage from the brain to the heart. ICP monitoring is invasive, requiring surgical insertion of a ventricular catheter, but it is indicated in severe TBI (Brain Trauma Foundation, 2007b). Ventricular catheters are both diagnostic and therapeutic, allowing drainage of CSF if ICP is too high. In general, CSF drainage is recommended if ICP increases to more than 20 mmHg (Chesnut et al., 2012). If CSF drainage alone is inadequate, then osmotic therapy with hyperosmolar agents (mannitol or hypertonic saline) should be considered. These hyperosmolar agents create an osmotic gradient that draws water across the BBB, thereby decreasing interstitial volume in the brain. Caution should be applied when using these agents, because they invariably trigger diuresis and may lead to an acute decrease in MAP.

MAP can be maintained with fluid infusion and the use of vasopressors. Normal saline is the fluid of choice to maintain euvolemia. Use of albumin is associated with a twofold increase in mortality and should be avoided (Chesnut et al., 2012). Infusion of excessive volume should also be avoided, because hypervolemia may cause further ICP elevations by raising central venous pressure and impeding venous return from the brain. In addition, hypertension may exacerbate intracranial hemorrhage.

44.4.1 Other targeted therapies

Many pharmacologic agents and therapeutic strategies have been tested in clinical trials for management of secondary insults after TBI. Unfortunately, no specific neuroprotective agent or strategy has been shown to improve outcome (Maas et al., 2008).

Hyperglycemia or hypoglycemia should be avoided. Hyperglycemia is associated with poor outcome after TBI and should be avoided. However, aggressive treatment of hyperglycemia with insulin infusions has the risk of iatrogenic hypoglycemia, which also leads to adverse outcomes. Glucose control remains a controversial issue in TBI and other critical care arenas. There is a lack of consensus on the exact range of target glucose levels. In general, it is recommended to avoid extremes of hyperglycemia or hypoglycemia.

Normothermia should be maintained. Prehospital hypothermia is strongly associated with poorer outcome after TBI (McHugh et al., 2007). However, hyperthermia has been postulated to exacerbate secondary inflammatory insults. There is no clear evidence, however, that hyperthermia leads to worsened neurological damage or outcome (Childs, 2008). Despite many clinical trials, neither therapeutic hypothermia nor antipyretic treatments have been shown to correlate with improved mortality or morbidity (Henderson et al., 2003; Sydenham et al., 2009). Hypothermia could potentially lead to coagulopathies, metabolic derangements, and increased infections, and is thus not recommended as routine practice.

Early use of antiepileptics may improve outcome. *Posttraumatic seizures (PTS)* may develop in up to 20% of patients with TBI (Temkin et al., 1990). Seizures increase cerebral blood flow and metabolic demand, which increases ICP and aggravates secondary brain injury (Vespa et al., 2007). Thus, early antiepileptic therapy is recommended, which can reduce the rate of early PTS, but does not prevent later development of PTS (Temkin, 2001).

Glucocorticoids may worsen outcome. Steroids were considered in the treatment of TBI due to postulated potential in reducing inflammation and edema. However, a large clinical trial showed that use of methylprednisolone in early TBI is associated with increased mortality without benefits (Roberts et al., 2004; Edwards et al., 2005). Thus, the use of glucocorticoids is not recommended.

44.4.2 Opportunities for rehabilitation and recovery posttraumatic brain injury

TBIs can produce lifelong impairments, and intensive rehabilitation is often warranted for recovery. Once an individual is considered medically stable, they may qualify for intense acute inpatient rehabilitation. To qualify for this type of rehabilitation, an individual must be able to tolerate intense therapy for a minimum of 3 h/day, 5 days/week. Each individual is assigned a healthcare team consisting of a medical doctor, case manager, nurse, neuropsychologist, physical therapist, occupational therapist, and speech therapist that are all trained in TBI management. Physical, cognitive, and psychosocial impairments are evaluated and treated by the healthcare team. Interventions such as activities of daily living, functional mobility tasks, communication, and memory activities are all used to promote recovery. The healthcare team establishes goals with the emphasis on returning the individuals back to life before injury.

TBI is often described as a silent epidemic that leaves individuals with lingering cognitive and psychosocial deficits throughout the individual's life span (Uhl et al., 2013). Family training is often required to help adapt to a

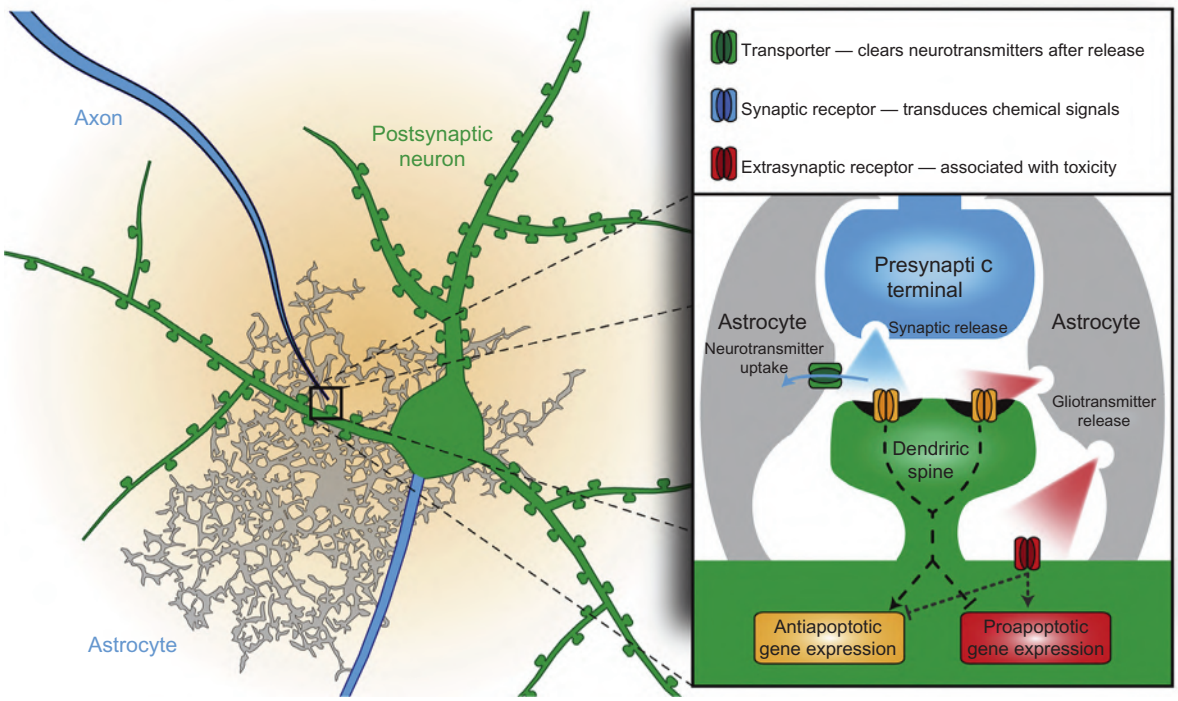

FIGURE 44.4 The synapse is a tripartite structure. Many synapses consist of three components: (1) a presynaptic site; (2) a postsynaptic site; and (3) a glial element. Presynaptic sites typically occur on axons and release neurotransmitters in an activity-dependent manner. Neurotransmitters bind to receptors on the postsynaptic site and elicit a cellular response. The activity of neurotransmitters is regulated by astrocytic processes, which express transporter proteins to uptake neurotransmitters such as glutamate. There is also emerging evidence that astrocytes may release neuroactive compounds (called "gliotransmitters") in an activity-dependent manner (called "gliotransmission"). Because of their peripheral location at the synapse, gliotransmitters from astrocytes tend to activate presynaptic and extrasynaptic receptors. Hyperactivation of extrasynaptic NMDA receptors (NMDARs) can lead to apoptotic cell death.

new way of life after injury. The healthcare team will work closely with family members, suggest modifications, and teach new strategies to help ease the transition back home.

44.5 Cognitive impairments

Memories are stored in our synapses, the connections between neurons. Our ability to form, recall, and lose memories requires the formation, maintenance, and loss of specific synapses. In general, we consider episodic memories to be stored in two broad areas: the hippocampus (for short-term memories) and the cerebral cortex (for working and long-term memories). Memories are required for us to build our seamless conscious experiences, in which we perceive senses, plan actions, and experience emotions through the activity of a vast network of synaptic connections within and between the cortex and hippocampus (Fig. 44.3). As such, the loss of neurons and synapses is attributable to the cognitive decline observed in many pathological states, including TBI. In fact, impaired short-term memory and attention are characteristic of the majority of TBIs (Faul et al., 2010). As discussed above, the superficial location of the cerebral cortex makes it particularly vulnerable to TBI.

44.5.1 Neuronal loss

Neurons are polarized cells with a number of extensive projections. In general, neurons receive information at their *dendrites* or cell body and transmit information along their *axons* (Fig. 44.4). Although a vast oversimplification, it is generally true that a greater number of neurons and synapses can allow a greater diversity of information to be encoded. Thus, the loss of neurons disrupts information processing and accounts for the behavioral changes observed in several neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, and Huntington's disease).

Cell death can occur through a variety of mechanisms, such as necrosis, autophagy, apoptosis, and necroptosis. Of these, necrosis and apoptosis are highly relevant to TBI. *Necrosis* is uncontrolled cell death caused by acute disruption of the plasmalemma. In TBI, necrosis can occur through shearing forces that rip apart axons or osmotic imbalance due to hyperactive ionic conductances that occur during excitotoxicity. Conversely, *apoptosis* is

a form of programmed cell death and is controlled by a variety of intracellular signaling cascades. Briefly, apoptosis occurs when contents in the intermembrane space of the mitochondria leak into the cytoplasm. Notable examples include cytochrome c, Smac/DIABLO, and Omi/Htra2, which lead to the activation of *caspases*—cysteine proteases that cleave a variety of intracellular contents. Through autoproteolytic activation, caspases create a positive feedback loop of proteolysis that inevitably leads to cell death. Accordingly, the cell contains the Bcl-2 family of proteins, which regulate mitochondrial permeability and caspase activity. A detailed description of apoptosis and its regulation by Bcl-2 proteins is beyond the scope of this chapter. Readers interested in additional details about the regulatory role of Bcl-2 proteins in apoptosis should read the work by Youle and Strasser (2008).

The loss of neurons through necrotic and apoptotic pathways may explain some of the cognitive impairment that occurs after TBI. Markers of necrosis and apoptosis are detectable in human cases and animal models of TBI. In controlled cortical contusion rat TBI models, cortical and hippocampal degeneration is evident within hours of injury, and the magnitude of degeneration is related to TBI severity (Sutton et al., 1993). Hippocampal neuron loss is relevant to memory impairments and a feature of the majority of severe TBI cases. A postmortem analysis by Kotapka et al. (1992) demonstrates how extensive this loss can be—most patients with severe, fatal TBI lose more than two-thirds of their neurons in region CA1 (Kotapka et al., 1992). The loss of neurons in the hippocampus after TBI correlates with cognitive impairments in human patients and rodent TBI models (Hicks et al., 1993; Bigler et al., 1997; Tate and Bigler, 2000).

44.5.2 Synapse loss

In addition to neuronal loss, more subtle alterations in neuronal and synaptic function are observed after TBI. Because these changes do not involve stark cell loss, they are potentially reversible. Before discussing alterations, it is important to give an overview of synaptic structure and function. Synapses can be either electrical or chemical. We focus on chemical synapses (called synapses for the rest of the chapter) because they are, by far, the most common type of synapse. Synapses are incredibly small structures, with a diameter of 300 ± 150 nm and a gap of only 20 ± 2.8 nm (Ribrault et al., 2011). By and large, synapses comprise three parts: a presynaptic site, a postsynaptic site, and a glial element (Fig. 44.4).

Presynaptic sites are typically found along axons and are the site of neurotransmitter release. *Postsynaptic sites* are typically found on dendrites and cell bodies. The postsynaptic site contains a high density of neurotransmitter receptors that bind neurotransmitters and transduce the signal into a cellular response. Postsynaptic sites that occur at *dendritic spines* are very plastic to allow the storage of new information (i.e., memory formation). Finally, the *glial element* is an astrocytic projection that is involved in buffering the concentration of ions and neurotransmitters in the extracellular space. Astrocytic projections play an important role in preventing neurotransmitters from leaking outside the synapse or building up to toxic levels—preventing astrocytic uptake of the excitatory neurotransmitter glutamate leads to lethal, convulsive seizures.

All three of these synaptic elements are affected by TBI (Fig. 44.5). Presynaptic sites are destroyed by DAI, in which rotational forces cause a shearing of axons. DAI can be measured in vivo using *diffusion tensor imaging (DTI)*. Loss of white matter (i.e., axons) scales with TBI severity—moderate-to-severe TBI patients have obvious, global white matter damage, whereas mild TBI patients have more subtle alterations. Axonal damage measured by DTI also correlates with cognitive impairments (Kraus et al., 2007). Postsynaptic alterations are also obvious after TBI. In controlled cortical impact mouse TBI models, dendritic spine density is reduced in the cortex and hippocampus within days of the injury (Gao et al., 2011; Winston et al., 2013). The loss of dendritic spines reduces the number of postsynaptic sites, thus reducing neuronal excitability and restricting release from the presynaptic site. Thus, TBI can compromise synaptic function at presynaptic and postsynaptic sites in the absence of stark neuronal loss. Finally, glial elements are disrupted after TBI. One of the most reliable alterations is, in fact, reactive gliosis. In reactive gliosis, microglia and astrocytes alter their morphologies in order to quarantine damaged areas of the nervous system and recruit macrophages to remove cell debris. Pronounced reactive gliosis may be damaging to neurons due to glutamate excitotoxicity (see below).

It is not clear whether glial damage occurs before, after, or simultaneously with neuronal damage. However, it is likely that both cell types experience acute injury simultaneously, but neurons are more vulnerable to damage. Consistent with this notion, markers of neuronal injury are detected earlier than markers of glial injury in the CSF of children after TBI (Berger et al., 2002).

The brain is a dynamic organ, and dendritic spines are perhaps the most dynamic structures in the brain, showing the ability to rapidly retract and grow in an experience-dependent manner (Schubert and Dotti, 2007). In a unilateral fluid percussion injury rat TBI model, the excitatory synapse marker PSD95 is downregulated in the cortex and hippocampus (Campbell et al., 2012a). In harmony with this PDS95 reduction, the density of dendritic spines, particularly in the cortex, decreases acutely (<24 h) after

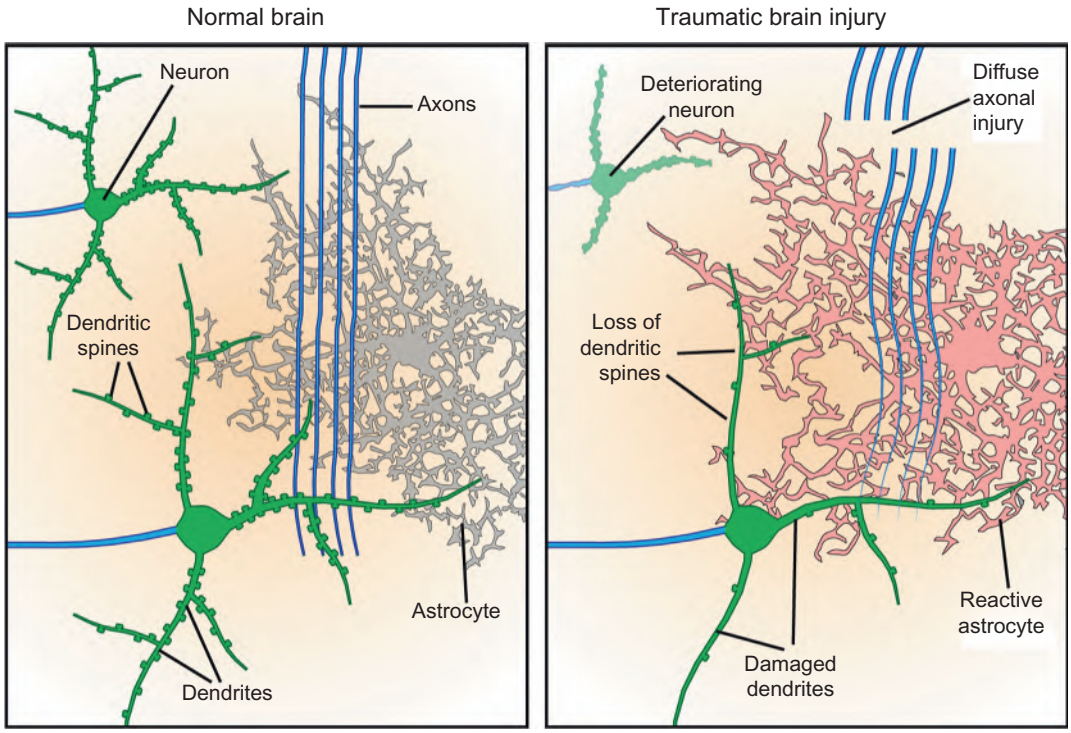

FIGURE 44.5 Traumatic brain injury causes cell death, synapse loss, axon damage, and astrocyte reactivity.

fluid percussion injury (Campbell et al., 2012b). However, after 1 week, the density of dendritic spines recovers. Interestingly, hippocampal neurons have a higher density of dendritic spines 1 week after TBI compared with controls. The significance of this is not clear, although the authors speculate that elevated spine density in the hippocampus may explain the epileptogenic activity reported after TBI.

44.5.3 Seizures

Seizures occur in many cases of brain injury, including stroke, neoplasm, neurodegenerative diseases, infection, and all types of traumatic injuries. The risk of PTS increases with the severity of TBI. The 30-year cumulative incidence of PTS is 2% for mild TBI, 4% for moderate TBI, and >15% for severe TBI (Annegers et al., 1998). This figure can rise much higher. For example, over 50% of Vietnam veterans with penetrating head injuries suffered posttraumatic seizures (Salazar et al., 1985).

There are likely to be several mechanisms of seizure induction following TBI (Fig. 44.6). Brain swelling or mass lesions (i.e., hematoma or neoplasm) increase intracranial pressure and can deform the membranes of neurons. Physical deformation of neurons leads to delayed depolarization, making them more excitable. Neurons may also express different ion channels and transporters following injury, leading to an increase in excitability. A notable example is the sodium-potassium-chloride cotransporter (NKCC1), which imports one Na^+, one K^+, and two Cl^- to help establish appropriate ionic concentrations across the membrane. Following TBI, NKCC1 is expressed at higher levels. Elevated NKCC1 increases intracellular Cl^- levels, which decreases the inhibitory effects of GABA and increases the excitability of neurons.

Additionally, TBI can lead to an inflammatory response that can directly and indirectly stimulate neurons. Neurons are stimulated by a variety of proinflammatory cytokines that are released following nervous system injury. Proinflammatory cytokines also trigger reactive gliosis, in which microglia and astrocytes undergo morphological and functional changes in order to respond to injury. Reactive microglia can release (1) proinflammatory cytokines, which amplify inflammation, and (2) glutamate, which may lead to excitotoxicity (discussed below). Reactive astrocytes may also contribute to glutamate excitotoxicity through glutamate release or reduced uptake—there are inconsistent data of glutamate transporter function in reactive astrocytes. Glutamate toxicity may also be caused by glutamate released by T cells that invade the nervous system during the inflammatory response. In addition to proinflammatory cytokines and glutamate, neurons are also stimulated by an increase in extracellular K^+. Extracellular K^+ levels are tightly regulated by astrocytes, and impaired K^+ buffering by reactive astrocytes leads to epileptiform activity.

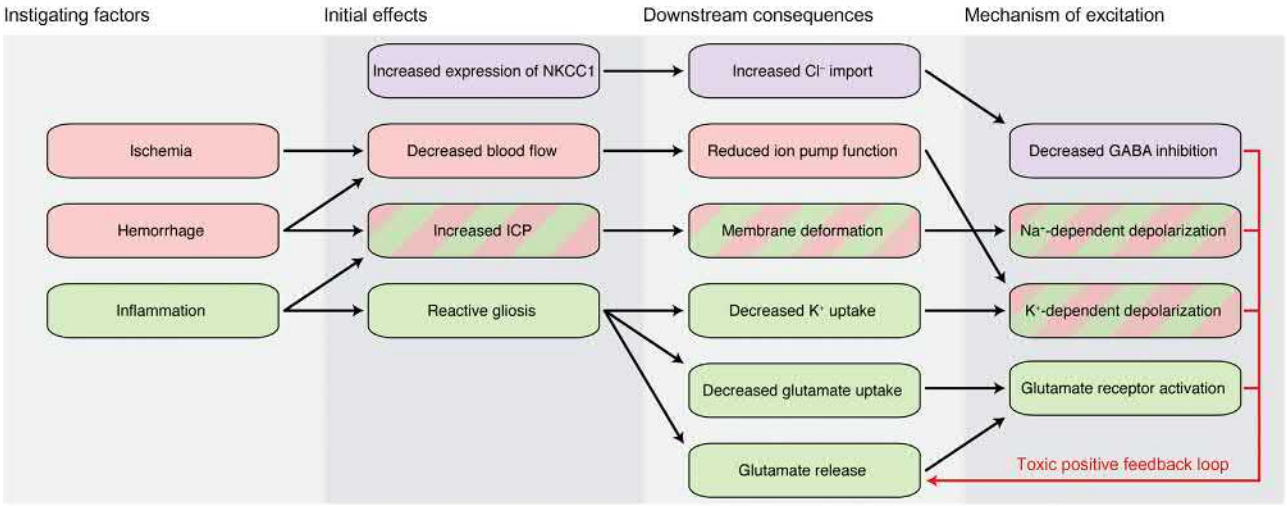

FIGURE 44.6 **Potential mechanisms of seizure induction.** TBIs can damage nervous (green) and vascular (red) tissues. Damage to any tissue produces an inflammatory response, which can cause brain swelling and reactive gliosis. Vascular effects include hemorrhage, which can create hematomas and increase ICP, and ischemia, which decreases blood flow to nervous tissue. Ischemia may be caused by increased ICP or vasospasm following injury. Neurons may also respond to injury by altering gene expression. One example includes an increase in the sodium, potassium, chloride cotransporter NKCC1 (purple). Alterations in gene expression, blood flow, ICP, and glial function increase neuron excitability through a variety of mechanisms. A few selected mechanisms are shown above. Hyperactive neurons can create a toxic feedback loop through the release of glutamate (signified by the red arrow), which drives another wave of neuronal activity.

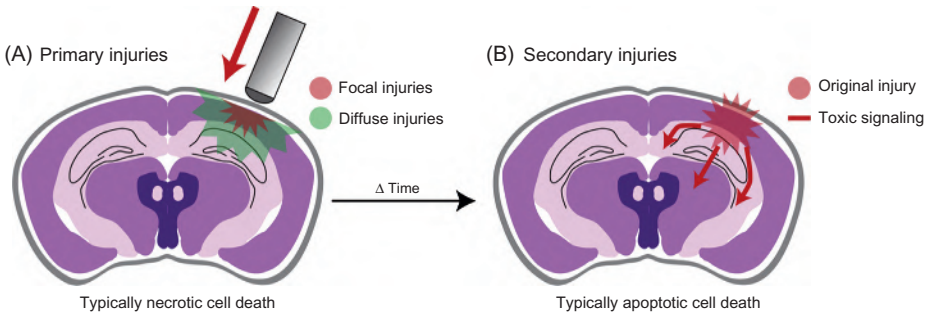

FIGURE 44.7 **TBI comprises several types of injury.** (A) Primary injuries result from the physical forces of the TBI. Focal damage is caused by contact forces that drive the brain into the skull, whereas diffuse damage is caused by inertial forces (linear and rotational acceleration of the head that shear axons). (B) Over time, secondary injuries emerge due to aberrant cellular signaling cascades that emanate from the initial site of damage.

44.6 Cellular mechanisms of primary and secondary injuries

Neurological injuries that result from a TBI are classified as focal versus diffuse and primary versus secondary (Fig. 44.7). Focal injuries occur in confined areas of the brain and are caused by contact forces to the head. Diffuse injuries occur over more widespread brain regions and are caused by inertial forces to the head. Primary and secondary injuries are distinguished based on their cause—primary injuries are caused directly by a TBI, whereas secondary injuries result from downstream effects of the original TBI. As a result, primary injuries occur immediately following the TBI and are associated with necrotic cell death. Secondary injuries may take hours to weeks to manifest and are generally attributable to apoptotic cell death. The physical and cellular basis for these injuries is discussed.

44.6.1 Necrosis

The mechanical force of a TBI causes immediate injury due to contact and inertial forces. Contact forces are the forces that prevent the head from moving after impact, which drives the brain into the skull. Even in the absence of skull fracture, movement along naturally occurring ridges and protuberances of the skull causes focal injuries to the cerebral cortex. Inertial forces are the linear and rotational accelerations of the head. Inertial forces cause axonal shearing, particularly at the junction of gray and

white matter, which have different densities. This accounts for the diffuse nature of injuries that result from inertial forces. The primary injuries caused by contact and inertial forces are not likely to be treatable due to the limited regenerative capabilities of the central nervous system (CNS). Instead, these injuries must be prevented through the use of protective equipment.

The DAI that occurs during primary injury impairs the propagation of signals from the cell body of neurons to presynaptic terminals. White matter tracts are particularly susceptible to shearing forces incurred during a TBI. DAI is only confirmed by microscopic analysis, although there are many in vivo imaging and detection methods that are used to assess axonal integrity. DTI is an MRI variant that measures the diffusivity of water molecules. In white matter, water is unable to diffuse freely and is restricted in certain directions. This directional restriction of diffusion is termed anisotropy. By measuring the fractional anisotropy of different brain regions, white matter maps can be created to trace bundles of axons. DTI can readily detect DAI in moderate-to-severe TBI patients, whereas mild TBI patients show some subtle alterations (Inglese et al., 2005; Kraus et al., 2007). Using in vivo microdialysis in patients with mild TBI (Glasgow Coma Score = 9), Petzold et al. (2011) monitored axonal injury by detecting neurofilament heavy chain, an intermediate filament found in axons. Extracellular levels of neurofilament heavy chain show two phases of elevation: a pronounced, immediate elevation and a delayed, longer lived elevation (Petzold et al., 2011). Presumably, these phases reflect primary and secondary neuronal loss: the marked, early elevation is likely due to axonal shearing, which spills out the intra-axonal contents, whereas the later elevation is due to neuronal loss through secondary injuries.

Axons in the CNS may be particularly vulnerable to rotational and translational forces due to the high compaction of myelin. Myelin is a lipid-rich substance that wraps around 0.3- to 2-mm-long segments of axons. The purpose of myelin is twofold: myelin insulates the axon to speed the conduction of action potentials and myelin reduces metabolic demand by decreasing the number of ions that move across the membrane during an action potential. Myelin in the peripheral nervous system (PNS) is moderately compact, providing some cushioning to allow peripheral nerves to withstand compression forces. PNS myelin is synthesized by Schwann cells, which produce an extracellular matrix (ECM) that contains proteins that support axonal regeneration, such as laminin-2 (Chen and Strickland, 2003)—this explains the minimal regenerative properties of the PNS. Myelin in the CNS, however, is highly compacted to allow a greater density of synapses. However, this comes at the cost of providing minimal cushioning in the event of compression forces. CNS myelin is synthesized by oligodendrocytes, which also secrete little to no ECM. As a result, when axons are severed, the myelin sheath does not have the appropriate growth signals to allow regeneration. In fact, CNS myelin contains factors that may actually prevent axonal regeneration (Huang et al., 2005). This lack of regenerative abilities in the CNS highlights the importance of preventing TBIs through the use of proper protective equipment.

44.6.2 Apoptosis

Hours or days after the primary injury, secondary injuries develop. Secondary injury is caused by the release of substances that alter synaptic function, blood flow, ionic and neurotransmitter homeostasis, metabolic function, and inflammatory signaling pathways. This section focuses on mechanisms of neuronal loss by secondary injuries; mechanisms of synaptic alterations are discussed in the next section.

As discussed, apoptosis is a highly regulated form of programmed cell death that culminates in the activation of caspases. Caspase 3 activation is detectable in the vicinity of cerebral contusions (Petzold et al., 2011), highlighting the importance of maintaining the integrity of the BBB. Disruption of the BBB is associated with TBI and a variety of neurodegenerative disorders (Fig. 44.2 and Chapter 48: Blood–brain barrier damage and dysfunction by chemical toxicity). A variety of other proapoptotic markers are detectable in the CSF of infants and children after severe TBI, including cytochrome c, caspase 1, and Fas. The increase in proapoptotic markers was greater in girls; however, the cause of this gender difference is not clear (Satchell and Lai, 2005).

The expression of prosurvival and proapoptotic genes in cortical neurons was measured using antisense mRNA (aRNA) amplification in individual cortical neurons with fragmented DNA (a sign of cellular damage). After 12 h, prosurvival genes (e.g., neurotrophins, TrkB, and superoxide dismutase) are downregulated in injured neurons. After 24 h, the levels of many prosurvival gene expressions return to normal, although by this time the proapoptotic genes caspase-2 and bax are upregulated. The study by O'Dell et al. (2000) shows that genetic alterations occur rapidly after TBI and compromise cell health.

If apoptotic pathways are activated after TBI and play a role in the ensuing impairments, then apoptosis inhibitors should offer some therapeutic benefits. Continuous administration of the calpain inhibitor AK295 15 min after injury rescues motor deficits in a fluid percussion injury TBI rat model. However, only marginal improvements were observed in memory function (Saatman et al., 1996). Along these lines, inhibiting formation of the mitochondrial permeability transition pore with cyclosporine A has therapeutic benefits in a variety of TBI models, although utility in human TBI patients has yet to be demonstrated (Okonkwo et al., 1999; Sullivan et al., 2000;

Mazzeo et al., 2009). Taken together, these data show a clear connection between TBI and activation of apoptotic pathways.

44.6.2.1 Glutamate dysregulation and excitotoxicity

The principal excitatory neurotransmitter in the CNS is glutamate. There are two classes of ionotropic glutamate receptors—NMDA and non-NMDA receptors. Non-NMDA receptors desensitize rapidly and show no voltage gating. NMDA receptors (NMDARs), however, desensitize slowly and are only active when the cell is depolarized enough to remove a Mg^{2+} ion that blocks the ion channel. Glutamate levels are tightly regulated by glutamate transporters expressed in neurons and astrocytes.

Persistent glutamatergic activity leads to excitotoxicity through two routes: necrosis and apoptosis (Fig. 44.8). In both routes, persistent glutamatergic input depolarizes the cell and allows hyperactivation of NMDA receptors, which desensitize much more slowly than non-NMDA receptors and thus allow more ions to enter the cell. Acutely, as sodium and calcium rush in through ionotropic glutamate receptors, they make the cell hyperosmotic. Acute swelling can lead to rapid necrotic cell loss. Alternatively, if the cell successfully reestablishes ionic imbalance across its membrane, it will deplete its ATP stores. To replenish ATP, mitochondrial activity increases, which elevates the production of free radicals (termed reactive oxygen species, ROS) that lead to oxidative stress. Both oxidative stress and sustained Ca^{2+} elevations can induce cell death by activating apoptotic proteases through formation of mitochondrial transition pores that allow the efflux of proapoptotic signaling molecules.

Nilsson et al. (1990) used microdialysis to measure changes in a variety of energy-related metabolites and neurotransmitters in a weight-drop rat TBI model. Two hours after TBI, extracellular glutamate levels increase in relation to TBI severity; mild TBI increases glutamate eightfold, whereas severe TBI increases glutamate 13-fold. Increases in glutamate coincide with higher levels of lactate, the end product of glycolysis (Nilsson et al., 1990). Because astrocytes display higher levels of anaerobic metabolism than neurons, these data suggest that after TBI, neuronal activity is upregulated, which leads to increased glutamate uptake and glycolytic activity in astrocytes in an attempt to buffer this excess glutamate. As discussed, increased levels of glutamate will eventually lead to intracellular calcium increases, which can lead to apoptosis or more subtle alterations in cellular/synaptic function. Readers interested in additional details about the role of glutamate excitotoxicity in TBI should read the work by Arundine and Tymianski (2004).

Because glutamate is a ubiquitous, excitatory neurotransmitter with potentially toxic consequences, the extracellular level of glutamate is tightly regulated. After presynaptic release, glutamate is rapidly taken up by astrocytes. As discussed, astrocyte processes surround

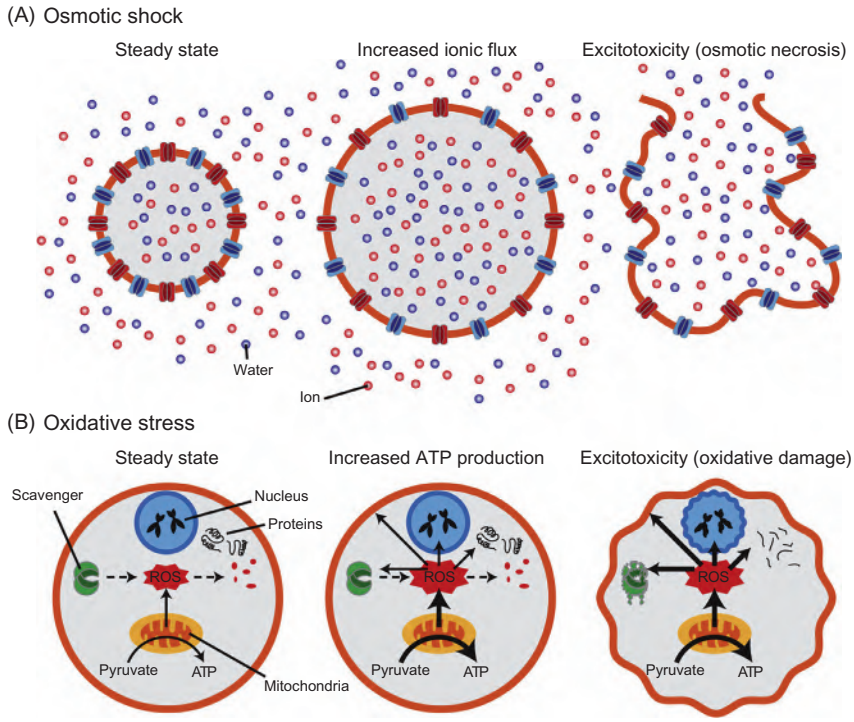

FIGURE 44.8 Excitotoxicity occurs through two routes. Excitotoxicity is caused by excessive glutamatergic signaling. (A) Continuous ionic flux increases the osmolarity of the cell. In extreme cases, this will drive enough water into the cell to cause osmotic necrosis. (B) If the cells survive the massive influx of ions, then ionic imbalance is reestablished in an ATP-dependent manner through a variety of ion pumps. As more ATP is generated through the TCA cycle and oxidative phosphorylation, ROS are produced. High rates of ATP production will produce toxic levels of ROS that cannot be scavenged by intracellular reducing agents [e.g., ROS, glutathione, and nicotinamide adenine dinucleotide phosphate (NADPH)]. ROS oxidize a variety of macromolecules to damage the DNA, proteins, and membranes of the cell. Ultimately, this oxidative damage results in apoptosis.

many synapses and play a role in buffering neurotransmitter levels. In the case of glutamatergic transmission, astrocytes regulate extracellular glutamate levels through high-affinity excitatory amino acid transporters (EAATs 1 and 2). For many years, astrocytes were viewed as morphologically simple cells due to their visualization using antibodies against the intermediate filament glial fibrillary acidic protein (GFAP). It is now clear that GFAP occupies a mere 10% of astrocytic volume and that astrocytes extend numerous tortuous processes to sample nearly all of their occupied volume, with essentially no overlap between adjacent astrocytes (Halassa et al., 2007). However, neighboring astrocytes are coupled to one another via gap junctions to create an extensive network with greater buffering capacity and potentially unappreciated signaling capabilities. Readers interested in additional details about astrocytic network functions should read the work by Giaume et al. (2010).

After injury, astrocytes and microglia undergo reactive gliosis, which is characterized by cellular hypertrophy and, in extreme cases, reentry into the cell cycle. Reactive gliosis is one of the most reliable markers of neurological damage and occurs within days of TBI. Although reactive gliosis is generally thought of as neuroprotective, cellular hypertrophy may lead to glutamate release from reactive microglia and astrocytes. Additionally, the epileptiform activity that occurs following TBI will cause neurons to release high levels of glutamate and increase metabolic demands. Because glutamate uptake is driven by ionic gradients that are established in an ATP-dependent manner, depletion of energy stores and reduced metabolic support will necessarily reduce glutamate uptake by neurons and astrocytes. Because of their peripheral location at synapses, glutamate released from astrocytes may be particularly toxic due to the activation of extrasynaptic NMDA receptors.

NMDA receptors are found at and away from the synapse. In general, synaptic NMDA receptors are considered neuroprotective, whereas activation of extrasynaptic NMDARs leads to apoptosis (Fig. 44.4). This may be a homeostatic adaptation that eliminates leaky synapses (i.e., those that allow glutamate to escape and active extrasynaptic NMDARs). The details of synaptic versus extrasynaptic NMDA receptor signaling are still not clear, although it does appear that these two pathways have opposing functions on prosurvival, CREB-dependent gene expression and prodeath, FOXO-dependent gene expression. Readers interested in additional details about the cytotoxic effects of extrasynaptic NMDA receptors should read the work by Hardingham and Bading (2010).

44.6.2.2 Oxidative stress

A commonly reported event that occurs after TBI is an increase in ROS from mitochondria. ROS are highly reactive free radicals (e.g., superoxide and hydroxyl radicals) that damage a range of macromolecules, including lipids, proteins, and DNA. Thus, cells maintain reducing conditions through production of NADPH and glutathione, which work with superoxide dismutase to neutralize ROS. Elevated levels of ROS are indicative of cellular stress and will eventually lead to cell death if left unchecked.

Bayir et al. (2002) published a comprehensive analysis of antioxidant reserve and oxidative damage after TBI. Early on, 1 day after TBI, CSF samples from children and infants with severe TBI show higher levels of the oxidative stress marker F2-isoprostane. F2-isoprostane levels normalized after day 2. However, levels of the antioxidant ascorbate are reduced immediately after TBI (day 1) and continue to decline afterward (up to day 7). Glutathione, an important reducing agent, levels show a biphasic response to TBI: glutathione is elevated immediately after TBI and declines after 5 days (Bayir et al., 2002).

A variety of antioxidant compounds have been used to scavenge ROS in rodent TBI models and human TBI patients (Dohi et al., 2006; Hall et al., 2010). Some ROS scavengers produce positive impacts on survival and neurological outcomes in larger phase III clinical trials (Marklund et al., 2001; Hall et al., 2010). Despite this, ROS scavengers are not currently used as a therapeutic option to treat TBI.

44.6.2.3 Cell-cycle reentry

Neurons are *postmitotic* cells; thus, they no longer undergo cell division. This loss of reproductive capabilities is attributable to their complex cellular morphology. To undergo cell division, neurons must retract their intricate dendritic and axonal arbors—an impossible task. Neurons that reenter the cell cycle invariably fail to complete the task and die. Because of this, neurons actively suppress cell-cycle reentry. This suppression is removed in several neurodegenerative disorders, including TBI.

Cyclin-dependent kinase inhibitors, such as flavopiridol, prevent cell-cycle progression. In a lateral fluid percussion injury rat model of TBI, the expression of genes associated with DNA injury and cell-cycle progression are upregulated. These genetic alterations appear to play a causative role in neuronal cell death, because intracerebroventricular injection of the cell-cycle inhibitor flavopiridol reduces neuron loss. Flavopiridol also reduces the activation of astrocytes and microglia, although it is unclear whether cell-cycle reentry occurs in neurons, glia, or both, or whether neuronal dysfunction and glial dysfunction synergize during TBI (Di Giovanni et al., 2005).

44.7 Potential mechanisms of synaptic impairment

Synapses are maintained in part through neurotrophic signaling. Neurotrophins are a class of proteins that activate receptor tyrosine kinases (Trk receptors) to regulate the growth and maintenance of synapses. Cell growth is stimulated by neurotrophins through their activation of Rho GTPases, a diverse family of proteins that (in general) positively regulate the stability and growth of actin filaments and microtubules. Through their positive impact on cytoskeletal organization, neurotrophins induce the outgrowth of dendrites and axons in a variety of neurons. The importance of neurotrophic support on synaptic maintenance is illustrated in mice that lack brain-derived neurotrophic factor (BDNF), a neurotrophin implicated in learning and memory. BDNF knockout mice develop normal dendritic arbors but fail to maintain them (Gorski et al., 2003). Because dendrites are important postsynaptic sites, this reduction in dendritic arborization reduces the number of possible synaptic contacts.

As discussed, neurotrophic expression is downregulated in neurons with DNA fragmentation after TBI (O'Dell et al., 2000). However, whole-brain analysis shows upregulation of neurotrophins within the same time frame in a controlled cortical impact TBI rat model (Oyesiku et al., 1999). Another study using in situ hybridization shows that some neurotrophins increase after mild TBI (e.g., BDNF), whereas others (e.g., NT-3) decrease (Hicks et al., 1999). These data suggest that nondamaged neurons and glia upregulate their expression of neurotrophins to offset the decreased expression in injured neurons. However, upregulation of neurotrophins may be deleterious as high levels of neurotrophins activate the low-affinity $p75^{NTR}$, which is linked to proapoptotic and synapse-degenerating signaling pathways.

The loss in postsynaptic sites is regulated by enzymes that control the polymerization state of actin. Work by Campbell et al. (2012a) provides mechanistic insights into the postsynaptic alterations that occur in a fluid percussion injury rat TBI model. Within 18 h after injury, PSD95 levels in the cortex and hippocampus decline, indicating the loss or shrinkage of postsynaptic sites. Reduction of PSD95 is preceded by an increase in the activity of cofilin—an actin-severing enzyme. When cofilin is activated, it breaks apart actin filaments and causes the shrinkage of dendritic spines (i.e., excitatory postsynaptic sites). Normally, cofilin is held in an inactive state through phosphorylation of Ser3, which disallows interactions between cofilin and actin. However, TBI increases the activation of the protein phosphatase calcineurin, which dephosphorylates and activates cofilin. Because calcineurin activity is regulated by calcium−calmodulin, these results suggest that calcium dysregulation after TBI may cause the loss of excitatory synapses through cofilin-dependent actin depolymerization.

44.8 Pathological hallmarks of Alzheimer's disease in traumatic brain injury

44.8.1 Alzheimer's disease: Aβ and tau

Alzheimer disease (AD) is a progressive dementia that is classically characterized by amyloid plaques, tau neurofibrillary tangles, and brain atrophy. Amyloid plaques are extracellular aggregates composed mostly of aggregated forms of Aβ. Although plaques are easily detected postmortem, cognitively normal elderly individuals can have high levels of plaques (Buckner et al., 2005). Additionally, a population of Japanese AD patients with a specific mutation in Aβ (E22Δ) exhibited dementia in the absence of plaques (Tomiyama et al., 2008). Therefore, many experts in the field have moved away from the amyloid cascade hypothesis proposed by Hardy and Higgins (1992) and instead believe the soluble oligomer hypothesis, in which diffusible Aβ oligomers (AβOs) attach at or near synapses and alter synaptic function (Hardy and Higgins, 1992; Wilcox et al., 2011). A similar movement has occurred with regard to the microtubule-associated protein tau: soluble tau oligomers are viewed as more toxic than insoluble tau neurofibrillary tangles. Finally, it is absolutely critical to remember that the best correlate with dementia severity is not brain atrophy, but rather synapse loss (Terry et al., 1991). Thus, although most view AD as being typified by plaques, tangles, and atrophy, these are antiquated views.

Aβ is derived by proteolytic processing of the amyloid precursor protein (APP) by a group of proteases called *secretases* to produce a number of soluble peptides with diverse cellular functions. Aβ is produced when APP is cleaved outside the membrane by *β-secretase* (now identified as BACE-1) and within the membrane by *γ-secretase*. The strongest support for a causative role for Aβ in AD progression comes from human genetics. There are several mutations and duplications in APP and γ-secretase that can lead to heritable familial AD (FAD). FAD-association mutations can increase Aβ production (Swedish, Flemish), promote Aβ polymerization into toxic forms (Osaka and Arctic), or shift the metabolism of APP to produce more Aβ that is 42 amino acids long (Florida, London), two amino acids longer than normal and far more prone to polymerization. It is important to note that one mutation exists that actually decreases the risk of development of AD (Icelandic) by disrupting Aβ production (Jonsson et al., 2012).

Based on studies using tau knockout mice, it appears that Aβ induces synaptic loss and eventually cell death by

structural and functional modifications to tau. Normally, tau is localized in axons, where it binds to and stabilizes microtubules. However, when tau is phosphorylated, it leaves the microtubule and activates a protein phosphatase 1 (PP1) through its phosphatase activating domain (PAD). PAD likely acts as an autoregulatory mechanism to limit the time that tau spends away from the microtubule and to prevent tau from becoming hyperphosphorylated—tau hyperphosphorylation is associated with AD progression. In fact, when tau is hyperphosphorylated in AD, it no longer interacts with axonal microtubules and translocates to the cell body and dendrites.

Tau is phosphorylated by a number of kinases, but glycogen synthase kinase 3β (GSK3β) appears to be highly relevant to AD. AβOs indirectly activate GSK3β, thus causing tau phosphorylation. Phosphorylated tau activates PP1 through its PAD. PP1 dephosphorylates both tau and GSK3β, which keep the kinase active and inevitably lead to tau hyperphosphorylation. The functional consequence of tau hyperphosphorylation is "synaptic starving," in which impaired axonal transport disrupts the delivery of critical proteins and energetic substrates to the synapse, eventually leading to synapse loss (Mandelkow et al., 2003). Alterations in intracellular trafficking are likely due to prolonged GSK3β activation (Kanaan et al., 2011). GSK3β phosphorylates the motor protein kinesin, which delivers vesicular cargo to presynaptic and postsynaptic sites. Phosphorylation by GSK3β causes kinesin to dump its cargo, which, in the case of chronic GSK3β activation, can lead to depletion of synaptic components. Thus, the tau−PP1−GSK3β-positive feedback, which may be initiated by Aβ, inevitably leads to tau pathology and synaptic dysfunction.

44.8.2 Aβ in traumatic brain injury

Amyloid pathology is observed in rodent TBI models and human TBI patients. In fact, diffuse amyloid plaques are present in the brains of professional boxers with TBI at levels comparable with those observed in AD patients (Roberts et al., 1990). APP, BACE-1, presenilin (a component of γ-secretase), and Aβ accumulate in the terminal portion of severed axons after TBI (Chen et al., 2004). By concentrating APP and the secretases together, Aβ production is favored. Increased Aβ production leads to oligomer formation and eventually synaptic dysfunction and loss. Accordingly, Aβ accumulation increases with repetitive head injury and correlates with injury severity (Uryu et al., 2002; Tran et al., 2011). However, attenuating Aβ production with a γ-secretase inhibitor does not prevent the loss of dendritic spines after a controlled cortical impact TBI (Winston et al., 2013). This suggests that although TBI may promote the onset of AD pathology, acute synaptic damage occurs through alternative routes. It is likely that elevated Aβ levels may play a role in the chronic effects of TBI on synaptic structure and function, although this is purely speculative.

44.8.3 Tau in traumatic brain injury

Because TBIs increase Aβ burden, and because Aβ acts upstream of tau, TBIs also induce tau pathology. Brains from TBI and AD patients show tau hyperphosphorylation at the same amino acid residues (Schmidt et al., 2001), and both occur in cortical pyramidal cells, albeit in different subpopulations (Hof et al., 1992). Neurofibrillary tangles are found in TBI patients years after a single TBI (Johnson et al., 2012). As expected, axonal transport appears to be impaired in mouse models of TBI (Säljö et al., 2000).

Tau pathology appears in a delayed fashion after TBI. A single mild TBI does not increase tau pathology after 3 weeks in hTau mice, which express human tau isoforms. Instead, multiple TBIs are required to observe tau pathology, although astrocyte reactivity is apparent 3 weeks after a single TBI (Ojo et al., 2013). Because of the axonal localization of tau, one likely cause of tau pathology in TBI is DAI, which occurs when delicate axons are torn apart during rapid head acceleration (Elson and Ward, 1994). However, based on the time required for tau pathology to become apparent, it is likely that increased Aβ production drives tau hyperphosphorylation through alterations in intracellular signaling.

44.9 Concluding remarks and future directions

TBI is a multifactorial and sporadic neurodegenerative syndrome that is caused by contact and inertial forces that lead to primary injuries that are focal or diffuse, respectively. Early primary injuries cause necrotic cell loss. The surviving cells have compromised cellular function or support, leading to secondary injuries. Secondary injuries lead to aberrant cell signaling, impaired synaptic function, and apoptotic cell death. The mechanisms of apoptotic cell death have multiple factors and include glutamate excitotoxicity, oxidative damage, and reduced neurotrophic support. In the absence of marked cell loss, synaptic loss is also observed after TBI. Synaptic impairments are the result of impaired neurotransmitter homeostasis, which leads to aberrant calcium signaling. Pathological overlaps between TBI and AD also contribute to the loss of synapses and neurons.

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Chapter 45

Neurological effects and mechanisms of blast overpressure injury

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45.1 Introduction

Traumatic brain injury (TBI) is a major cause of disability in civilian and military populations. In civilians, TBIs are usually caused by unintentional falls, especially in elderly persons. On the other hand, military personnel are more likely to experience TBI from blast or explosive injuries, as blast injuries account for the majority of casualties during active combat. For example, blast injuries accounted for 236 of the 269 casualties treated at a US Army echelon II medical facility between October 1, 2003 and June 30, 2004 during Operation Iraqi Freedom, with 111 of those 236 blast injuries involving the head or multiple body areas (Murray et al., 2005). This has led to *blast TBI (bTBI)* being deemed the "signature injury" of the Iraq and Afghanistan conflicts.

The incidence of probable TBI in deployed military personnel is estimated to be between 11−23% (Lindquist et al., 2017). This estimate far exceeds the reported incidence of 0.33% for diagnosed TBIs in soldiers deployed to Afghanistan and Iraq between September 11, 2001 and September 30, 2007 (Wojcik et al., 2010). Regardless of the actual incidence rate for TBI, it is clear that a large number of military personnel suffer from TBI. Between January 1, 2000 and March 31, 2018, 383,947 US military service members were diagnosed with a TBI (Defense and Veterans Brain Injury Center, 2019). The majority of TBIs were classified as mild (82%), followed by moderate (10%), penetrating (1.4%), and severe (1.1%). TBIs were most frequently reported in members of the Army (58% of all TBIs).

Blast or explosive injury is the most common cause of head injury in veterans, particularly those with high combat exposure. In Iraq and Afghanistan veterans, bTBIs accounted for over 60% of all TBIs (Wojcik et al., 2010). The cause of bTBIs is most commonly *improvised explosive devices (IEDs)*. Based on the medical records of soldiers who suffered blast injuries during Operation Iraqi Freedom (from March 2004 to February 2007), IEDs accounted for nearly 70% of all injuries, followed by mortars (8%), rocket-propelled grenades (5%), and landmines (5%), with the remaining 12% left unclassified (Dougherty et al., 2011). The increased use of IEDs has been attributed to the higher overall instance of TBI and bTBI in Iraq compared to Afghanistan.

45.2 Blast waves and mechanisms of injury

Classically, TBI has been divided into two groups: (1) *penetrating TBI (pTBI)* and (2) *closed TBI (cTBI)*. pTBI involves a foreign object penetrating the skull and entering the brain parenchyma, causing physical damage to nerve cells, fiber tracts, and blood vessels. In addition to physical damage from the foreign object, the subsequent hemorrhage, edema, and cerebrospinal fluid (CSF) leakage amplifies nerve cell loss in pTBI. cTBI involves sudden head acceleration that leads to brain deformation within the intact cranium. Contact between the brain and skull can lead to physical trauma to nervous tissue. In addition, head rotation produces shearing forces that sever fiber tracts. Depending on the severity of the cTBI, hemorrhage may or may not occur.

bTBIs are complex and can include elements of both pTBI and cTBI depending on the types of injuries (see Table 45.1 and Fig. 45.1). For example, pTBI may be caused by secondary blast injuries in which shrapnel penetrates the skull. cTBI may be caused by the blast wave itself (primary) or when blunt objects strike the head (secondary or tertiary). It is important to note that although primary injuries likely represent the unique

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TABLE 45.1 Blast injuries types.

Type of injury	Cause of injury
Primary	Direct interaction between blast pressure wave and organs
Secondary	Impact of physical objects propelled by blast pressure wave
Tertiary	Impact with physical objects when victims are flung by blast pressure wave
Quaternary	Burns or toxic fume inhalation

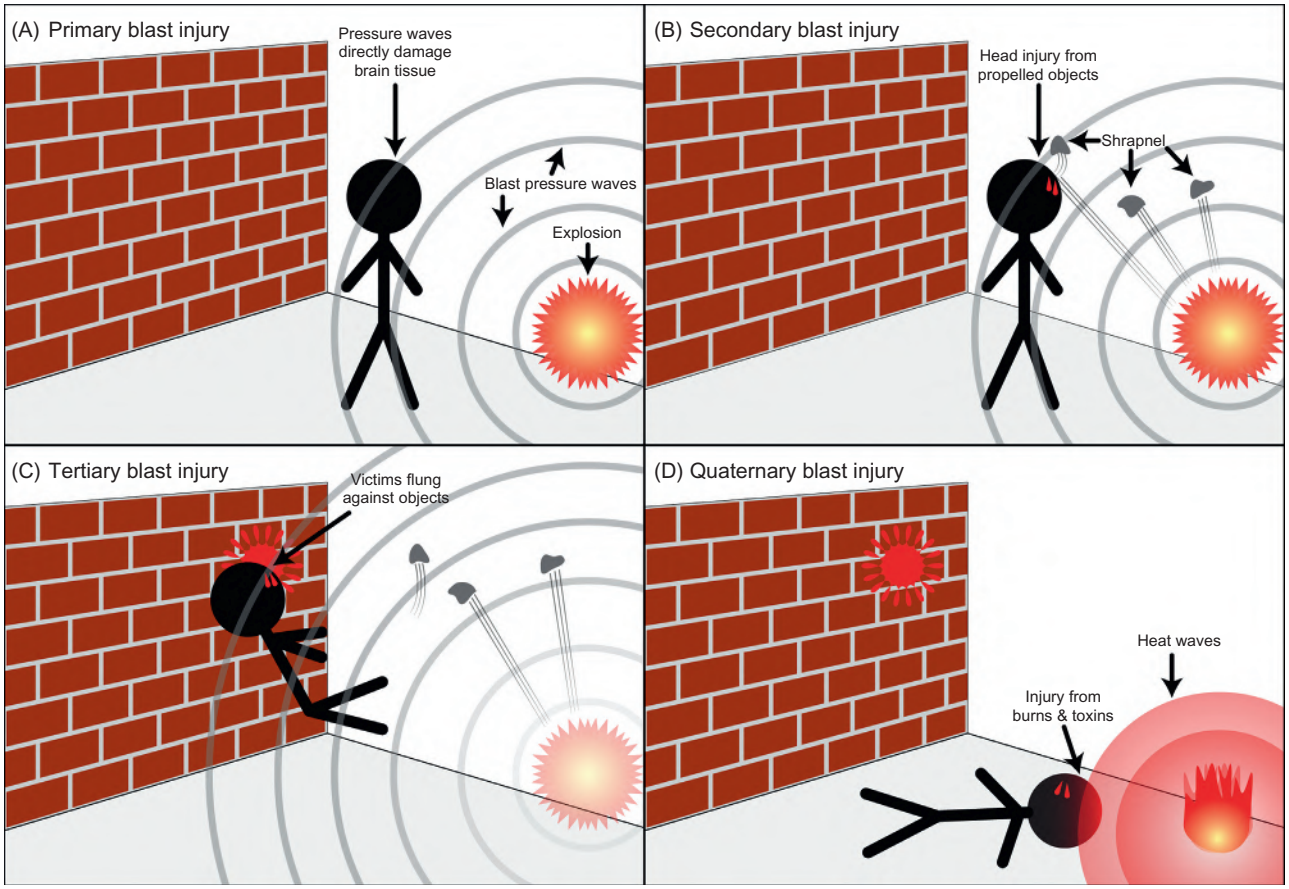

FIGURE 45.1 Explosive devices cause multiple types of injuries. (A) Primary injuries are caused by tissue deformation from blast pressure waves. (B) Secondary injuries are caused by shrapnel and other objects propelled by blast pressure waves. (C) Tertiary injuries are caused when victims are flung against objects, such as a wall, by blast pressure waves. (D) Quaternary injuries arise from heat and toxic fumes produced by the explosion.

aspect of blast TBI, primary injuries almost never occur in isolation. Adding to the complexity of bTBIs is the fact that blast pressure waves affect other bodily regions besides the head, leading to peripheral effects that may amplify brain damage.

45.2.1 Pressure waves

Explosive devices employ violent chemical reactions to transfer energy into the surrounding medium (i.e., air). This energy transfer compresses air molecules, increasing air pressure and temperature to produce *pressure waves*. Pressure waves travel through the air at supersonic speeds that exceed the characteristic wave speed of air, leading to compression of pressure waves to form *shock waves* that include a sharp high-pressure phase followed by a negative pressure phase (see Fig. 45.2). This characteristic change in air pressure (P) over time (t) is described by the Friedland equation: $P(t) = P_s * e^{-t/t^*}\left(1 - (t/t^*)\right)$, where $P(t)$ is the air pressure at any given time, P_s is the

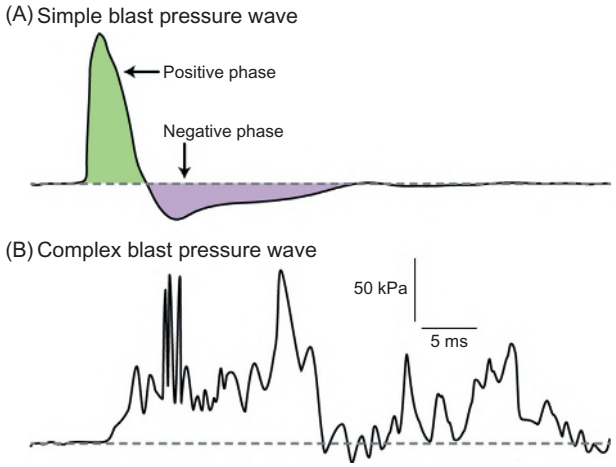

FIGURE 45.2 **Pressure waves compress to form shock waves.** (A) The idealized pressure waveform over time exhibits a sharp peak of positive pressure followed by a negative pressure phase. (B) Pressure waves reflecting off solid surfaces create complex blast waves.

peak pressure, and t^* is the time at which air pressure becomes negative. The behavior of spherical shock waves is an open field and well characterized, with the peak pressure change decaying nonlinearly as a function of distance (d). Idealized decay based on spheric expansion is equal to the cube of distance, while empirical measurements estimate the decay of open field overpressure waves to be $P_s = d^{-1.2}$ (Reed, 1972).

The behavior of pressure waves in enclosed spaces is more complicated and varies on a case-by-case basis. Pressure waves reflected off solid surfaces—such as walls, ceilings, or the skull—can merge to form a *complex wave field*. This can expose victims to multiple amplified pressure waves. This, along with the closer proximity to the explosion, likely explains the roughly sixfold increase in mortality reported for blast injuries sustained in buses compared to open-air bombings (Leibovici et al., 1996). Furthermore, different numbers of casualties were reported during the March 11, 2004, bombings of commuter trains in Madrid, Spain. The two trains with open doors had far fewer casualties (29 and 17 deaths) than the two trains with closed doors (64 and 67 deaths) (Turégano-Fuentes et al., 2008). The Madrid bombings presumably all occurred with similar distances to the explosive device, providing strong evidence that exposure to complex blast waves is associated with higher mortality than open-field blasts.

45.2.2 Mechanism of primary injury

The transduction of pressure into the brain could occur through two possible routes: direct transcranial propagation or indirect propagation through bodily fluids (e.g., blood or CSF). The prevailing view favors transcranial propagation of pressure waves. Only ~3% of the pressure delivered to the abdomen makes it to the brain (Säljö et al., 2008). However, in rat models, protection of the abdomen and thorax with a Kevlar vest reduces mortality and axonal injury (Long et al., 2009). These findings could be explained by species differences in vasculature or differences in the blast models used. It may also be the case that peripheral injury exacerbates TBI through effects on cardiopulmonary function, which may explain the increased stroke risk reported in TBI patients (Burke et al., 2013).

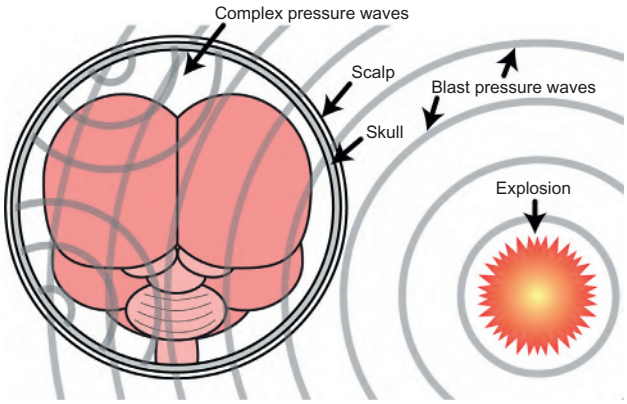

FIGURE 45.3 Pressure waves rebound off the skull to produce complex pressure waves.

Primary injuries from pressure waves are caused by physical tissue deformation. Pressure waves move the particles in the medium they pass through. When pressure waves encounter tissue or pockets of fluid or air within tissues, they cause movement. Blasts waves, particularly at close range, deliver nonuniform energy to tissues that they encounter, thus creating nonuniform tissue movement and deformation. Nonuniform movement of the brain generates shearing forces that can sever axons. Blast waves may rebound off the skull or protective helmet, creating complex wave fields and exacerbating injury (Fig. 45.3). Air-filled organs, such as the lungs, are particularly susceptible to high-pressure waves. In fact, the most common fatal primary blast injury is *blast lung* (i.e., pulmonary barotrauma). The consequences of blast injury are discussed further in the next section.

45.3 Clinical features of traumatic brain injury

All types of TBIs share many of the same clinical features. In fact, patients suffering bTBI and cTBI have nearly identical abnormalities on neuropsychological tests (Mac Donald et al., 2014). Currently, there is no strong evidence that bTBI produces distinct changes in cognition compared to other TBI mechanisms. As such, I will

TABLE 45.2 TBI severity.

Severity	Loss of consciousness	Posttraumatic amnesia	Neuroimaging	Glasgow rating
Mild	<0.5 h	<1 h	Normal	13–15
Moderate	0.5–24 h	1–7 days	May be abnormal[a]	9–12
Severe	>24 h	>7 days	Abnormal	4–8

[a]*Abnormal neuroimaging includes signs of intracranial bleeding, skull fractures, brain swelling, diffuse axonal injury, or brain atrophy.*

TABLE 45.3 Using the GCS to assess head injury severity.

Behavior	Response	Score
Eye opening	Open and blinking spontaneously	4
	Open to verbal stimuli	3
	Open to painful stimuli	2
	No response	1
Verbal	Aware of self, time, and location	5
	Slight confusion	4
	Inappropriate replies	3
	Incomprehensible replies	2
	No reply	1
Motor	Follows commands	6
	Appropriate purposeful withdrawal from pain	5
	Normal flexion in response to pain	4
	Abnormal flexion in response to pain	3
	Extension in response to pain	2
	No response	1

Classification:
Coma: 3.
Severe head injury: 4–8.
Moderate head injury: 9–12.
Mild head injury: 13–15.

briefly describe the common clinical features of TBI as these are covered further in Chapter 44, Clinical and cellular aspects of traumatic brain injury.

45.3.1 Common clinical features of traumatic brain injury

TBIs can produce clinical features as mild as transient confusion or as severe as coma. TBIs are classified into one of three categories based on neurological signs and neuroimaging data: mild TBI, moderate TBI, and severe TBI (see Table 45.2). Mild TBI is characterized by: (1) loss of consciousness <30 min, (2) posttraumatic amnesia <1 h, and (3) normal neuroimaging (e.g., no contusion, hematoma, or skull fractures). *Moderate TBI* is characterized by: (1) loss of consciousness between 0.5–24 h, (2) posttraumatic amnesia between 1–7 days, and (3) neuroimaging that may or may not be normal. *Severe TBI* is characterized by: (1) loss of consciousness >24 h, (2) posttraumatic amnesia >7 days, and (3) abnormal neuroimaging. These classifications are made almost exclusively based on subjective neurobehavioral exams, such as the *Glasgow Coma Scale (GCS)* (Table 45.3). Neuroimaging data (e.g., CT or MRI) are only occasionally used for diagnostic purposes.

TABLE 45.4 Clinical symptoms of traumatic brain injury.

Type	Symptoms	Notes
Sensory	Headache	Common. May persist or worsen in moderate or severe TBI
	Neck pain	–
	Dizziness	Common. May persist in moderate or severe TBI
	Nausea or vomiting	Common and brief. May persist or worsen in moderate or severe TBI
	Ringing in the ears	–
	Blurred vision	–
	Sensitivity to light or sound	–
	Numbness in limbs	More common in moderate or severe TBI
	A bad taste in the mouth	–
Motor	Reflexes	May be slow. Absent or abnormal reflexes may occur in moderate or severe TBI
	Dilated pupils	More common in moderate or severe TBI. May occur in one or both eyes
	Slurred speech	More common in moderate or severe TBI
	Weakness in limbs	More common in moderate or severe TBI
	Loss of coordination	More common in moderate or severe TBI
Cognitive	Confusion and difficulty concentrating	More common in moderate or severe TBI
	Memory loss	May be transient. More common in moderate or severe TBI
	Agitation	More common in moderate or severe TBI
	Loss of consciousness	Typically brief. Lasts for minutes to hours in moderate or severe TBI
	Seizures	More common in moderate or severe TBI
	Sleep abnormalities	Fatigue is more common with mild TBI. Difficulty waking from sleep may occur in moderate or severe TBI

Common symptoms of mild TBI include headache, fatigue, sleep disturbances, sensory abnormalities, dizziness, mood disturbances, confusion, inability to concentrate, amnesia, and seizures (see Table 45.4). Because mild TBIs are transient and may produce subtle impairments that arise after the initial injury, they may go undiagnosed. This is especially true in combat scenarios where nonlife-threatening injuries may be examined less thoroughly or dismissed. Undiagnosed TBIs may place victims at increased risk of additional TBIs, as they are less likely to be removed from combat. This is especially problematic, as the ill effects of nonlethal head injuries appear dose-dependent. Veterans with multiple head injuries are more likely to experience PTSD (relative risk = 1.56−3.21), depression (relative risk = 1.06−1.85), suicidal ideation (relative risk = 1.07−3.10), and pain (relative risk = 1.08−1.66) than veterans with a single head injury (Lindquist et al., 2017). Furthermore, multiple head injuries slows the recovery from TBI.

More severe forms of brain injury may lead to seizures. Seizures occur in >20% of patients with moderate to severe TBI within the first week following injury (Vespa et al., 1999). Seizures occur following a variety of brain injuries, including stroke, neoplasm, neurodegenerative diseases, infection, and all types of traumatic injuries. This suggests a common underlying pathological mechanism in all states of injury. Likely candidates include neuroinflammation and reactive gliosis, which may increase neuronal excitability through physical deformation of the membrane or increased extracellular concentrations of K^+ or glutamate (see Chapter 44: Clinical and cellular aspects of traumatic brain injury).

TBIs increase the risk of dementia or long-term cognitive dysfunction. Moderate and severe TBI are well-known risk factors for dementia, increasing the risk between two- and fourfold. The relationship between mild TBI and dementia is not yet clear, especially in cases with no loss of consciousness (Ramalho and Castillo, 2015).

Repeated head injuries can create a condition called *chronic traumatic encephalopathy (CTE)*, which is a sporadic tauopathy that causes dementia in patients with a history of head trauma. bTBI has been linked to CTE and mouse models exhibit characteristic alterations in tau structure. However, it remains unknown whether bTBI presents any elevated risk of dementia compared to other forms of head injury.

45.3.2 Distinct clinical features of blast traumatic brain injury

45.3.2.1 Blast lung

bTBI typically involves injury to additional areas beyond the head. Bodily injury is far more common in bTBI than pTBI and cTBI due to the mechanism of injury. A projectile or blunt object that strikes the head may completely spare the rest of the body from injury. On the other hand, blast pressure waves spread to potentially injure any unprotected area of the body. Consistent with this, blast lung is the most common fatal primary blast injury. Signs of blast lung include apnea, bradycardia, hypotension, chest pain, and hemoptysis. These signs usually occur immediately after blast injury, although they may take as long as 48 h to emerge. Damage to lung vasculature is visible by chest X-ray as a characteristic butterfly pattern.

45.3.2.2 Hearing loss, tinnitus, and visual impairments

The ears are another air-filled organ susceptible to blast injury. Accordingly, hearing loss and tinnitus are more common in soldiers suffering from bTBI compared to other forms of TBI (Lew et al., 2007). The most common injury to the middle ear from pressure waves is tympanic membrane rupture. Rupture of the tympanic membrane is associated with the loss of consciousness following bTBI (Xydakis et al., 2007). As such, one should suspect bTBI in victims of blast injury with hearing loss.

In addition to hearing loss, visual impairments can occur following bTBI. These can occur from damage to the eye, ocular nerve, or visual cortices. The likelihood of suffering an ocular or visual disorder increases with the severity of bTBI (Dougherty et al., 2011).

45.3.2.3 Postconcussive syndrome and posttraumatic stress disorder

Mild blast exposure can create a collection of symptoms referred to as *postconcussive syndrome (PCS)*, shell shock, or blast concussion. The symptoms of PCS overlap considerably with mild TBI listed in the previous section, and PCS can be caused by any head injury. One important aspect of PCS is the fact that it can be triggered years after injury, much like *posttraumatic stress disorder (PTSD)*. In fact, PTSD shares many features with PCS, and PTSD symptoms are more common in soldiers with blast injuries (Sayer et al., 2008). PTSD symptoms correlate better with PCS symptoms than injury mechanism (i.e., blast vs. nonblast), suggesting an important emotional component of PCS. While PCS and PTSD can mimic one another, PCS is suspected when physical and somatic symptoms are present. The relationship between blast severity and PCS is unclear, although additional head injuries slow recovery from PCS.

45.4 Human neuropathology of blast traumatic brain injury

As with clinical features, the neuropathological hallmarks of bTBI are similar to those seen with pTBI and cTBI. The lack of distinct neuropathological consequences in bTBI compared to other forms of TBI may be due to our extremely limited data from humans. Alternatively, the considerable overlap between the neuropathology of different forms of TBI may simply reflect the fact that they all involve brain tissue damage. Regardless of the injury mechanism, tissue damage triggers an inflammatory response, reactive gliosis, and alterations in neuronal function. In this section, I will outline neuropathological features of TBI and highlight any unique aspects of bTBI. A brief discussion of the clinical management of blast TBI is provided. For further details on clinical management of TBI, see Chapter 44, Clinical and cellular aspects of traumatic brain injury.

45.4.1 Neuropathological features of blast traumatic brain injury

Blast injuries cause a variety of neurological effects, ranging from extremely mild to fatal (Hicks et al., 2010). A range of neuropathologies can be observed in cases of bTBI, including edema, contusion, hematoma, hemorrhage, blood−brain barrier (BBB) disruption, diffuse axonal injury, and cortical atrophy. None of these are unique to bTBI, although they appear to emerge with different time courses in bTBI. Brain swelling occurs within hours of injury, leading to dangerous increases in *intracranial pressure*, which may compress neural and vascular tissues, leading to brain herniation or ischemia (Ling et al., 2009). This rapid swelling appears to occur more commonly in bTBI than other TBIs. Swelling from blast injuries is treated by military neurosurgeons with decompressive craniectomies more commonly than for other types of TBIs.

Another characteristic feature of bTBI is vasospasm. *Vasospasm* is the sudden contraction of arterial walls,

which may lead to ischemia. Cerebral vasospasms are commonly caused by *subarachnoid hemorrhage (SAH)*, although traumatic vasospasm can occur in the absence of hemorrhage and appears to be unique in terms of temporal course and pathophysiology (Kramer et al., 2013). The peak incidence of vasospasm following sporadic SAH is 5−14 days, compared to 5−7 days for traumatic SAH. Traumatic vasospasm in the absence of SAH peaks around 1.25 days.

The *BBB* is a collection of tight junctions and transporter proteins in the cerebral vasculature that acts as a diffusion barrier to restrict entry into the central nervous system. The BBB maintains the integrity of the interstitial fluid, and leakage at the BBB can allow the invasion of neuroactive compounds (e.g., neurotransmitters) or peripheral cells (e.g., immune cells). BBB permeabilization occurs in a host of neurological conditions, including tumor growth, neurodegenerative conditions, and TBI. Increased BBB permeability has been reported in blast and nonblast TBI patients (Korn et al., 2005; McKee and Robinson, 2014). BBB permeabilization may be exacerbated by neuroinflammation that occurs following injury.

Mild bTBI patients have a greater degree of cortical atrophy in the frontal lobes than nonblast TBI patients, leading to a corresponding decrease in executive function (Clark et al., 2018; Eierud et al., 2019). The degree of cortical thinning is related to PTSD, depression, and postconcussive symptoms (Michael et al., 2015). Additional areas of cortical thinning include inferior temporal and insular regions. However, it should be noted that cortical thickness varies considerably within groups and cortical thickness preinjury is unknown in these studies. As such, long-term follow-up will be important in bTBI victims to determine whether cortical atrophy rates are any different following bTBI.

Another site of injury in bTBI patients is the cerebellum. Cerebellar white matter injury has been reported in a small sample of military personnel with mild TBI following exposure to a single blast (Mac Donald et al., 2013). Exposure to repeated blasts negatively correlates with glucose uptake measured by fluorodeoxyglucose-positron emission tomography in the cerebellum of patients with mild TBI, indicating cerebellar hypoactivity (Meabon et al., 2016). The cerebellum has well-established roles in motor output coordination, language, and working memory function. As such, damage to the cerebellum may contribute to a variety of impairments that occur following bTBI.

In addition to damaging specific brain regions, TBIs cause diffuse axonal injury, in which a subset of axons are severed due to shearing forces on axons. Diffuse axonal injury causes widespread changes in the functional connectivity between different brain regions by severing the axons of projection neurons that interconnect brain regions, which may explain the changes in cognition, memory, perception, and motor function that occur following TBI. *Functional connectivity* refers to the degree to which the activities of two regions correlate with one another, reflecting their ability to function as a part of a larger network to carry out complicated tasks that require multiple brain regions (e.g., memory). Alterations in functional connectivity have been reported in a variety of cortical and subcortical structures and appear to be heterogeneous between patients. As such, there is no bTBI signature for altered functional connectivity.

45.4.2 Clinical management

The severity of blast injuries can be reduced with protective head and body equipment. This, along with advances in medical care, has led to the lowest kill:wounded ratio for soldiers in modern history, with fewer than 1 in 10 wounded dying. This high survival rate necessitates improved acute and long-term clinical care for TBI patients. Fortunately, the majority of TBI cases are mild and full recovery is expected to occur within 3 months following injury for 85% of victims—the other 15% develop PCS. Unfortunately, only about half of the patients with a single mild TBI are free of cognitive impairments after 3 months (Meabon et al., 2016). Patients with multiple head injuries have a poorer prognosis and an increased risk of long-term neurological defects (e.g., dementia).

Blast injuries are complex and often involve injuries to multiple body systems. The clinical management of TBI on the battlefield involves first controlling bleeding and ensuring proper airway and circulatory function. Next, the level of head injury is assessed using the GCS (see Table 45.3). Higher levels of injury necessitate more immediate evacuation and elevation of the level of care.

Once off the battlefield, more detailed assessments of injury and further treatments can be performed. CT scans can help identify intracranial hemorrhage, skull fractures, or pronounced increases in ICP. As discussed above, swelling is a common feature of the acute phase of blast TBI. Swelling is managed with intravenous hypertonic saline, mild hypothermia, and decompressive craniectomy. Although decompressive craniectomies can effectively treat elevated ICP, complications arise in greater than half of all cases and are more likely in patients who are older or have more severe injuries (Moon and Hyun, 2017). Potential complications of decompressive craniectomy include infection, CSF accumulation, hematoma expansion, and syndrome of the trephined (i.e., severe headache, dizziness, irritability, seizures, pain or discomfort at the surgical site, and psychiatric symptoms).

45.5 Animal models of blast traumatic brain injury

A variety of animal models exist for the different types of TBI (Xiong et al., 2013). bTBI can involve multiple types of injuries to a variety of body systems, as well as a host of physical parameters that may be impossible to replicate in the lab (e.g., complex wave fields). Add to this the sparsity of human data, and it becomes difficult to validate animal models of blast TBI. That said, mouse models of TBI all show signs of brain damage. As in any experimental approach, models with a greater degree of control over injury parameters have reduced physiological relevance, while models that aim to reproduce the conditions of blast injury may exhibit more variable degrees of injury.

The most commonly used animal models of TBI include fluid percussion, controlled cortical impact (CCI), weight drop injury (WPI) models, and blast overpressure injury (BOI) models. *Fluid percussion injury (FPI)*, *CCI*, and many *WDI* models are open models, meaning they involve a craniotomy in order to open a window in the skull and expose the cerebrum to external forces that displace and deform brain tissue. In FPI, the external force is generated when a pendulum is dropped to strike a reservoir of fluid, creating a fluid pressure pulse that is delivered to the dura. In CCI, the external force is generated by a pneumatic or electromagnetic device that drives a rigid impactor onto the dura. In WDIs, small weights are dropped from varying heights onto the exposed dura (open models) or skull (closed models). Open models reliably delivery mechanical energy to the cerebrum, allowing them to reproduce secondary injuries of bTBI with less variability than closed models. FPI, CCI, and WDI produce many of the neuropathological hallmarks of TBI, including intracranial hemorrhage, edema, axonal injury, BBB dysfunction, and gray matter atrophy. However, craniotomies are not benign and likely influence the results from these models.

BOI models use pressurized gas or miniature explosives to deliver blast pressure waves ranging from 20 to 350 kPa to the animal. Pressure waves can be generated using blast tubes, which contain a reservoir of air and a long tube to direct the blast separated by a diaphragm—diaphragm thickness determines blast pressure. Air is pumped into the reservoir until the diaphragm ruptures, producing a blast pressure wave that travels out of the blast tube. Alternatively, small explosives can be discharged at specified distances from anesthetized animals in order to deliver blast pressure waves. Experimental blast pressure waves reproduce the idealized pressure waveform (i.e., sharp peak of positive pressure followed by a negative pressure phase) and can be confined to the head with the use of Kevlar vests.

BOI is a more relevant model for bTBI than FPI, CCI, or WDI for two reasons. First, BOI does not require craniotomy and is thus a true closed TBI model. Second, the use of gas pressure waves accurately reproduces the physical parameters of primary blast injuries—other TBI models only reproduce secondary injuries, which are not specific to bTBI. For these reasons, I will focus my discussion on data from BOI models.

Animal models of bTBI reproduce many of the neuropathological hallmarks of human TBI, including vasospasm, edema, contusion, axonal injury, hemorrhage, reactive gliosis, and neuroinflammation. The neuropathological effects of BOI depend on the amplitude of the blast pressure wave (Long et al., 2009). Each experimental set-up must be calibrated in order to determine the lethal dose of blast pressure, so the absolute values of blast pressure waves may not translate well between different studies. At lower blast pressures, neuropathology may be absent. At moderate blast pressures, diffuse axonal injury occurs in the absence of overt cell loss. Subtle impacts on the vasculature can occur at moderate blast pressures, such as increased BBB permeability (Readnower et al., 2010). At higher blast pressures, more obvious neuropathologies emerge, including cortical degeneration, necrosis, inflammation, reactive gliosis, and hemorrhage.

The behavioral effects of BOI include reduced motor function, impaired sensorimotor reflexes, and learning and memory deficits as measured by maze navigation and novel object recognition. An important negative outcome of TBI in humans is the onset of mood disturbances. Mood disturbances can be detected in nonhuman animals by measuring a variety of anxiety-related behaviors, which typically involve hiding behaviors (e.g., avoiding well-lit areas or the center of an enclosure). Anxiety-related behaviors can be elicited by multiple exposures to extremely mild blast pressures, consistent with reports from veterans who suffered bTBIs. Remarkably, the learning and mood abnormalities that occur in BOI can be reversed by the mGluR2/3 antagonist BCI-838 in a dose-dependent manner (Perez-Garcia et al., 2018). These data suggest that elevated glutamate signaling may play a role in the PTSD symptoms associated with bTBI. Indeed, glutamate is elevated in the CSF of patients with head injuries (Yamamoto et al., 1999).

BOI has detrimental impacts on cardiopulmonary function, including acute apnea, bradycardia, and hypotension (Long et al., 2009). These likely contribute to the increased mortality rates observed following blasts, as encasing the body in a Kevlar vest reduced the cardiopulmonary effects and mortality of blast pressure waves. Whether this is a strength or weakness of the model depends on the experimental set-up and research question. Although the peripheral effects of blasts may increase injury variability, they also increase physiological relevance.

45.6 Biomarkers of blast injury

Given the subtle and transient nature of neurological deficits that occur following mild TBI, diagnosis can be challenging. This is especially true on the battlefield, where medical care providers may lack the time or resources necessary to assess mild TBIs. One appealing option is to use fluid biomarkers to assess the level of injury (Zetterberg and Blennow, 2016). Fluid biomarkers are molecules (i.e., proteins or RNAs) that can be measured in biological fluids such as serum or CSF.

I will discuss fluid biomarkers for TBI in general for two reasons. First, there is considerable overlap in the neuropathology and clinical symptoms that occur in blast and nonblast TBI. In other words, bTBI is clinically indistinguishable from other forms of TBI. Second, there is limited information of bTBI-specific biomarkers and little expectation for there to be unique biomarkers for blast injury. Markers of neuron death, reactive gliosis, demyelination, and neuroinflammation will appear regardless of injury mechanism. For example, elevated CSF levels of the microtubule-associated protein tau are found in patients with TBI, dementia (including Alzheimer's disease, Parkinson's disease, vascular dementia), stroke, brain tumor, infection, and hydrocephalus (de Bont et al., 2008; Franz et al., 2003; Hu et al., 2017; Kaerst et al., 2013; McKhann et al., 2011). From this, it seems that current fluid biomarkers are more likely to be useful for screening the level of injury, rather than determining the underlying cause of injury. Because the clinical utility of fluid biomarkers has not yet been determined, this section will merely survey the broad categories of biomarkers currently identified in TBI patients and mouse models.

45.6.1 Serum and cerebrospinal fluid protein biomarkers

Serum and CSF are commonly used biological fluids for obtaining biomarkers, and they each have their own advantages. Serum is easier to sample than CSF, while CSF has a smaller volume than serum, has lower protease activity than serum, and more directly contacts nervous tissue. Because of serum's greater volume (i.e., dilution), potential proteolytic degradation, and lack of free diffusion with interstitial fluid in the brain, serum biomarkers may be less sensitive to subtle or mild nervous system injury than CSF biomarkers.

Obtaining a sample of CSF is more difficult than obtaining a blood sample and, rarely, can produce adverse effects such as headache. CSF is usually sampled by lumbar puncture, although CSF can also be obtained through intracranial catheters in cases of severe TBI. Lumbar and intracranial CSF have different protein compositions, so it is important to note the source of CSF when interpreting these data.

45.6.1.1 Biomarkers of neuronal injury

Several different types of serum protein biomarkers have been identified in TBI (Agoston and Elsayed, 2012). The first class of protein biomarkers is those related to neuron death. When neurons die, their intracellular proteins are no longer confined by the plasma membrane. Neuronal intracellular proteins must also cross the BBB, which is presumably damaged, in order to enter the blood. The following proteins have been detected in TBI patients and animal models: neuron-specific enolase (NSE), tau, neurofilament heavy subunit, secretagogin, ubiquitin C-terminal hydrolase (UCH)-L1, α2 spectrin, and spectrin breakdown products.

NSE is among the most commonly studied biomarkers for TBI. Serum and CSF levels of NSE are elevated following severe TBIs and have been observed in BOI models (Pleines et al., 2001; Svetlov et al., 2010). Higher levels of serum and CSF NSE may be an indicator of poor prognosis (Böhmer et al., 2011; Žurek and Fedora, 2012). However, serum NSE is not sensitive enough to detect mild TBI, as mild TBI patients with and without intracranial lesions can only be distinguished based on serum NSE with a sensitivity of 56% and a specificity of 77% (Wolf et al., 2013). Despite its name, NSE is also highly expressed in erythrocytes, making it possible for hemolysis to contaminate serum samples.

45.6.1.2 Biomarkers of glial injury

The second class of protein biomarkers is those related to glial cell damage. As with neurons, the detection of intracellular proteins in extracellular fluids is a marker of cell death. Biomarkers of glial injury include S100β, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP). S100β and GFAP are commonly used markers of reactive astrocytes. MBP is a marker of demyelination and oligodendrocyte damage.

Elevated serum and CSF levels of S100β, GFAP, and MBP have been reported in TBI patients and BOI models (Böhmer et al., 2011; Gyorgy et al., 2011; Svetlov et al., 2010; Žurek and Fedora, 2012). Glial markers of injury may also have prognostic value, as CSF S100β and serum GFAP levels are significantly higher in nonsurvivors than survivors of severe TBI. Compared to S100β and GFAP, MBP levels rise later (>72 h) and remain elevated for longer (up to 2 weeks).

45.6.1.3 Biomarkers of inflammation

The final class of protein biomarkers is those related to inflammation. Biomarkers of inflammation include proinflammatory cytokines such as tumor necrosis factor (TNF) α, interleukins (ILs), and interferons (IFNs). Proinflammatory cytokines are released from glia following injury to stimulate reactive gliosis and recruit immune

cells to the site of injury. As such, elevated serum and CSF levels are expected to occur following injury. Indeed, elevated serum and CSF levels of proinflammatory cytokines have been reported in TBI patients (Woodcock and Morganti-Kossmann, 2013). The level of proinflammatory cytokines has prognostic value. For example, in severe TBI patients, elevated serum IL and TNFα levels correlate with increased ICP and decreased cerebral perfusion (Stein et al., 2011).

Neuroinflammation appears to be an early component of TBI, rising within minutes following injury. In animal models of bTBI, serum TNFα levels peaked earlier than tau or GFAP levels (Liu et al., 2014). Inhibition of TNFα synthesis prevents neuron loss and reactive gliosis in a WDI mouse model of TBI (Baratz et al., 2015). These data are consistent with cytokine measurements from postmortem human brain tissue, showing elevated TNFα, IL-6, and IFN-γ levels within minutes following TBI (Frugier et al., 2010). Taken together, these data suggest that neuroinflammation is an early instigating factor in neurotrauma.

45.6.1.4 MicroRNA biomarkers

There is considerable enthusiasm for the use of microRNA (miRNA) biomarkers in a variety of diseases. miRNAs are short, noncoding RNAs that shape the gene expression profile in cells by interacting with 3′ untranslated regions in messenger RNAs. miRNAs are important gene expression regulators in neurons and are implicated in the induction of neural cell fate, development of neural circuits, and maintenance of neuron function. In fact, roughly 70% of all known miRNAs are expressed in the brain (Adlakha and Saini, 2014).

miRNAs are secreted from cells into biological fluids and are surprisingly stable. Hundreds of secreted miRNAs have been identified as potential biomarkers for TBI, although only a small fraction of these miRNA biomarkers have been reported in multiple studies. miR-21, miR-16, and let-7i are of particular interest as TBI biomarkers due to their ability to differentiate between mild and severe TBI. It should be noted that miRNA levels can be highly variable between samples due to the exponential nature of polymerase chain reaction and the lack of appropriate normalization techniques. Because of this, miRNAs are currently more relevant in the laboratory than the clinic. For an in-depth discussion of miRNA biomarkers, readers are directed to recent reviews (Atif and Hicks, 2019; Di Pietro et al., 2018).

45.7 Concluding remarks and future directions

Blast injuries are often considered a signature injury for soldiers in active combat zones. Modern combat helmets and interceptor body armor vests improve survival after blast injuries, which necessitates the advancement of medical care for blast TBI patients. Civilian populations are not spared from blast injury, however, as explosive devices are commonly used during terrorist attacks.

Diagnosing bTBI may be difficult in many cases since the majority of bTBIs are mild and produce transient symptoms. Currently, fluid biomarkers are better suited to detect severe TBIs. The development of a biomarker signature for mild TBI may be especially useful on the battlefield, where time and resources are limited. This will require better normalization methods for protein and miRNA biomarkers. For example, NSE concentrations should be compared with free hemoglobin levels in order to estimate potential contamination by erythrocyte NSE in serum and CSF samples. Finally, the temporal sequence of biomarkers should be correlated with different levels of injury.

Currently, it is not clear to what degree bTBI is distinct from pTBI or cTBI. A large degree of overlap is expected between different types of TBIs in terms of pathophysiology, clinical symptoms, and fluid biomarkers. However, only two clear distinguishing features of bTBI have emerged so far. First, blast injuries may be accompanied by severe psychological distress that can create or exacerbate neurological symptoms of bTBI. This is likely due to the fact that bTBIs are typically acquired in military conflicts and terrorist attacks. Better diagnostic tools that discriminate between PTSD and bTBI are needed. The other key feature of bTBI is the peripheral injuries that occur due to the nature of blast pressure waves, which spread in spherical manner to affect the entire body. Animal models of blast TBI should account for the effect of primary blast injuries on the brain and body.

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Chapter 46

Genomics and proteomics in brain complexity in relation to chemically induced posttraumatic stress disorder

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46.1 Introduction

Posttraumatic stress disorder (PTSD) is an anxiety disorder rooted in an extremely traumatic experience, usually one that threatens severe injury or death. PTSD is characterized by symptoms in three primary domains: (1) reminders of the exposure (flashbacks, intrusive thoughts, and nightmares); (2) activation (hyperarousal, insomnia, agitation, irritability, impulsivity and anger); and (3) deactivation (numbing, avoidance, withdrawal, confusion, derealization, dissociation, and depression). Signs and symptoms can last beyond 1 month (even though such periods can occur long after the original traumatic exposure) and therefore reflect a persistent, abnormal adaptation of neurobiological systems to the stress of witnessed trauma. According to the World Health Organization (WHO), mental disorders account for 4 out of 10 of the leading causes of disability in developed countries, and epidemiological data suggest that 7.8% of people will experience PTSD in their lifetimes (Kessler et al., 1995; Roth et al., 2011; Gelernter and Sun, 2019). Clinical studies have shown a high prevalence of PTSD in war veterans, especially in those who suffered from chemical warfare. Based on symptom severity and persistence, PTSD is generally categorized into three types: acute, chronic, and delayed onset. In acute PTSD, symptoms normally last less than 3 months. In chronic PTSD, symptoms may last 3 months or more. In delayed onset of PTSD, symptoms first appear at least 6 months after the traumatic event. The estimated lifetime prevalence of PTSD is ~8% in the US population, with women (10.4%) twice as likely as men (5%) to experience PTSD. According to the same report, the prevalence ranges from 3% to 58% in high-risk groups, such as combat veterans and victims of violent crimes such as rape, sexual molestation, physical attack, and violence.

Current diagnostics for PTSD rely on subjective measures and patient recall, making it difficult to accurately diagnose the condition or differentiate its symptoms from those of depression or anxiety. The exact causes of PTSD and the long-term changes in the body, especially the brain, due to PTSD remain unknown. Identifying specific areas of the brain whose key functions are altered in association with a specific disorder is key to understanding the fundamental causes and sustaining factors of disease pathology. Recent advances in brain imaging technologies have allowed scientists to analyze the brains of patients afflicted with a variety of neurological disorders. Whole-brain imaging studies offered valuable information about the permanent changes and long-term effects of PTSD in the brain (Brown et al., 2014; Hull, 2002). However, some of these key brain areas are also involved in a number of closely associated brain disorders such as anxiety and depression (Kroes et al., 2011); and distinguishing subtle morphological changes between these closely related neurological disorders through brain imaging is extremely challenging. Additionally, whole-brain imaging studies can only provide a snapshot of the current morphological status, requiring the development of advanced methodologies that can predict the predisposition of an individual, mainly the alterations in genetic makeup, which serve as molecular signatures of PTSD. High-throughput genomics and proteomics approaches have been widely applied for identifying biomarkers for many diseases as well as characterizing the molecular toxicology of chemical agents (Gupta, 2014).

There are many questions that are relevant while trying to characterize the molecular fingerprint of a complex

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disease like PTSD. Determine whether there are changes in gene expression—that is, can the activity or output of particular genes—associated with the onset of PTSD after exposure to traumatic events or some environmental factors such as toxic chemical exposure affect the way our genes behave, the RNA they make, ultimately resulting in the alteration of the body's production of various proteins and hormones. Do those changes contribute to the development of PTSD? Can we identify specific variants in the genetic architecture involved in alterations in gene expression and associated with PTSD? Answers to these questions will help researchers understand what happens with our genes and the proteins they make and related basic biology as we are exposed to trauma and toxic environments. This, in turn, could educate new intervention efforts, including medication treatments that offset biological factors leading to PTSD's development. In this chapter, we briefly discuss the outcomes of current research in the anatomical changes in the brain, genomic factors leading to PTSD development, and recent advances in genomics, transcriptomics, and proteomics to understand PTSD at the molecular level, and develop early diagnostic tools and specific pharmacotherapy.

46.2 The effect of posttraumatic stress disorder on different regions of brain

Earlier brain imaging studies have identified a few key brain regions in which patients with PTSD appear to have altered stress response structure and function. The neurobiological systems regulating stress response pathways and the network of brain regions known to regulate fear and emotive behavior also seem to have a role in PTSD. Investigations to identify neurobiological markers for PTSD originally presumed that abnormalities were acquired as a consequence of traumatic experience. However, certain abnormalities in a patient with PTSD could simply represent preexisting conditions or functionally dormant pathology, which arise through trauma exposure and hence are detected thereafter upon investigation. Patients with PTSD showed drastic changes in the hippocampus and amygdala as well as cortical regions including the anterior cingulate, insula, and orbitofrontal region (Bremner et al., 2008; Shin et al., 2006). All these areas form a neural circuit mediating, among other functions, adaptation to stress and fear conditioning and changes in these circuits are considered directly related to the development of PTSD (Rauch et al., 2006).

46.3 The hypothalamic–pituitary–adrenal axis

The "headquarters" of the mammalian neuroendocrine stress response system is the hypothalamic–pituitary–adrenal (HPA) axis and, as such, it has been the main target for investigation in PTSD patients. Briefly, the HPA axis is made up of endocrine hypothalamic components, including the anterior pituitary and the adrenal glands as effector organs. Upon exposure to stress, neurons in the hypothalamic paraventricular nucleus secrete corticotropin-releasing hormone from nerve terminals in the median eminence into the hypothalamo-hypophyseal portal circulation, which stimulates the production and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH in turn stimulates the release of glucocorticoids from the adrenal cortex. Prolonged glucocorticoid exposure has adversarial effects on hippocampal neurons, involving a reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis (Fuchs and Gould, 2000).

Cortisol, secreted in the adrenal cortex in response to stress, is an informative biomarker that can be used to differentiate PTSD from normal patients. Outcome from a simulation study supported the hypothesis proposed by Yehuda and colleagues that high stress intensity and strong negative feedback loop may cause a hypersensitive neuroendocrine axis that results in hypocortisolemia in PTSD (Sriram et al., 2012; Yehuda et al., 1993). Also, low-dose dexamethasone suppression testing suggests that hypocortisolism in PTSD occurs due to increased negative feedback sensitivity of the HPA axis (Wingenfeld et al., 2007). In summary, these studies suggest that decreased availability of cortisol, due to irregular regulation of the HPA axis, may lead to abnormal stress reactivity and fear processing.

46.4 Hippocampus

Reduced hippocampal volume is a well-known hallmark feature of PTSD. Initial magnetic resonance imaging (MRI) studies determined smaller hippocampal volumes in Vietnam veterans with PTSD and patients with abuse-related PTSD compared with controls (Bremner et al., 1997). Therefore, the severity of trauma and memory impairments was related to the decrease in hippocampal volumes. However, reduced hippocampal volume has not been observed in children with PTSD (De Bellis et al., 1999). Additionally, proton magnetic resonance spectroscopy investigations further observed a reduction of the levels of *N*-acetyl aspartate (NAA), a marker of neuronal integrity, in the hippocampus of adult patients with PTSD (Villarreal et al., 2002). Hippocampal volume reduction in PTSD likely reflects a neurotoxic effect of repeated exposure to stress-increased glucocorticoid levels or glucocorticoid sensitivity, though decreased hippocampal volumes might also be a preexisting vulnerability factor for developing PTSD (van der Werff et al., 2013).

Convincing evidence suggested that smaller hippocampal volume in trauma-exposed persons resulted in them being diagnosed with more severe, unremitting PTSD (Gilbertson et al., 2002). In fact, early life stressors can affect hippocampal volume and predispose for the disorder (Yehuda et al., 2010). However, at this stage how trauma, per se, affects hippocampal volumetric (as well as other histopathological) measures, is not well understood. Intriguingly, functional neuroimaging studies have also shown deficits in hippocampal activation during a verbal declarative memory tasks in PTSD patients (Bremner et al., 2003). Both hippocampal atrophy and of functional deficits SSRIs notably, likely due to an increase of neurotrophic factors and neurogenesis (Nestler et al., 2002).

46.5 Amygdala

Investigators have suggested the idea that aberrant stress response and an enhanced amygdala-induced augmentation of emotional memories in PTSD subjects may be underpinned by abnormal amygdala functioning (Elzinga and Bremner, 2002) due to its functional role in mediating both stress responses and emotional learning. However, no clear evidence for structural alterations of the amygdala has been found in PTSD patients. Functional imaging studies have revealed amygdala hyperresponsiveness in PTSD subjects when stressful scripts (Hendler et al., 2003; Shin et al., 2004), cues, and/or trauma reminders were presented (Shin et al., 2006). On the other hand, PTSD patients show increased amygdala responses even to general emotional stimuli that were not trauma-associated (Shin et al., 2006) or subliminally threatening cues (Bryant et al., 2008; Hendler et al., 2003). Amygdala hyperactivity seems to be related with specific genetic traits (Hariri et al., 2002; Kilpatrick et al., 2007), hence it may likely have a role in developing PTSD. In fact, several investigations have found a positive relationship between activation of the amygdala and PTSD symptom severity (Shin et al., 2004).

PTSD patients show a hyperactive amygdala as a consequence of trauma and throughout the course of the disorder, hence reducing the activity of the amygdala could potentially prevent onset of the disorder or be therapeutic for those individuals who have already developed psychopathology. That led to the hypothesis that noradrenergic activity could promote amygdala-dependent fear memories. Indeed, researchers have speculated that adrenergic receptor antagonists could potentially reduce the severity of PTSD symptoms as adrenergic receptor modulators, such as prazosin (α1 receptor antagonist) (Raskind et al., 2007), clonidine (α2 receptor agonist) (Boehnlein and Kinzie, 2007; Strawn and Geracioti, 2008), and propranolol (β receptor antagonist) (Brunet et al., 2011; Pitman, 2011), have shown promising results as components of therapy for PTSD.

46.6 Cortex

The prefrontal cortex (PFC), and particularly the medial PFC, is highly involved in the extinction of fear memories (Sherin and Nemeroff, 2011) and in general in the inhibitory control of inappropriate cognitive and emotional responses that are mediated, in part, by the amygdala (Elzinga and Bremner, 2002). Given this large inhibitory role of the PFC over the amygdala, researchers have speculated that impaired PFC functioning may trigger amygdala hyperactivity and hence exacerbate emotional responsiveness. Actually, individuals with PTSD exhibit smaller volumes of the PFC, its major subregions, and specifically anterior cingulate cortex (ACC) (Woodward et al., 2006; Yamasue et al., 2003). Therefore, PTSD symptom severity could likely be related to these volumetric reductions (Zoladz and Diamond, 2013). Moreover, shape abnormalities (Corbo et al., 2005) as well as a decrease in NAA levels in the ACC (De Bellis et al., 2000) have also been reported.

However, recent evidence has suggested that volume loss in the PFC and ACC could also be a consequence of PTSD rather than a preexisting risk factor (Kasai et al., 2008). Functional imaging studies have demonstrated a reduced activity of the medial PFC in PTSD patients in response to stimuli, such as trauma scripts (Britton et al., 2005; Shin et al., 2004), combat pictures and sounds (Bremner et al., 1999), trauma-unrelated negative narratives (Lanius et al., 2003), fearful faces (Shin et al., 2005), and impaired performance on PFC-dependent tasks (Koenen et al., 2001). Finally, PTSD patients show impaired abilities to extinguish fear (Peri et al., 2000) and reduced activities of PFC regions during extinction trials (Bremner et al., 2005).

These individuals exhibited a reduced activity of PFC, or a complete failure to activate PFC brain regions during the presentation of trauma-associated stimuli (Britton et al., 2005; Shin et al., 2004), hence, it is likely that a reduced activation of PFC along with amygdala hyperactivity could lead to the development of the intrusive emotional thoughts and memories often experienced by PTSD patients. A reduced PFC activity could lead to greater governance of behavior by more primitive brain areas, such as the amygdala, impairing brain processes involved in adaptation, behavioral flexibility, and cognition. As for the volume decrease, it is still unclear whether the reduced PFC inhibition of lower brain areas, such as the amygdala, is a result of the disorder or a preexisting condition for developing PTSD. Investigations of twin brothers who were discordant for PTSD (Gilbertson et al., 2006) supported the hypothesis that reduced PFC

functioning is both a preexisting condition and a risk factor for the development of PTSD. Moreover, animal and clinical studies mainly suggest a complex interaction among early life stress (Karssen et al., 2007; Lyons et al., 2010), gene−environment influences (Garrido, 2011), and the well-described adverse effects of stress on PFC functioning in adulthood (Arnsten, 2009), which could interact to influence PTSD susceptibility and posttrauma expression. SSRI treatment has been shown to restore medial prefrontal cortical activation patterns (Shin et al., 2006).

Though the above findings using brain imaging studies advance our understanding of PTSD pathology, they fall short of identifying predispositions to the disease. The significant progress in genetics and genomics has generated approaches that screen full genome and genetic networks rather than individual genes. The recent move toward the "omics" fields, including genomics and proteomics, offers enormous opportunity to analyze genes, RNA transcripts, and protein expression patterns in diseased and normal tissues. Such approaches have equipped us with many advantages and made access to thousands of new molecular targets for disease screening and drug discovery. In the following pages we discuss the genomic and proteomic methods that are currently used to understand the molecular-level players in PTSD.

46.7 Understanding posttraumatic stress disorder: the genomics and proteomics way

The central dogma of biology coined by Francis Crick proposed that an organism's genetic information encoded in its DNA molecules is transcribed into RNA molecules (transcriptome), which are then translated to proteins (proteome) that facilitate particular biological functions. In addition to the transfer of information from genome to proteome, proteome functions are regulated by epigenetic mechanisms such as posttranslational modifications and regulation via noncoding RNAs (ncRNAs). The development of advanced techniques over past two decades has allowed the scientific community to take a closer look at the genome, transcriptome, and proteome of any tissue or organ, and proved to be invaluable in studying complex tissues such as the brain. Monitoring the specific activity of a genome by measuring mRNA expression levels of large gene sets can identify molecular profiles correlated to disease states, which may then be developed as diagnostic tools.

The human brain is one of the most complex biological structures, which consists of approximately 100 billion neurons and a nearly equal number of nonneuronal cells (Azevedo et al., 2009). Neurons that perform a specific function are assembled into circuits and the neurons within each circuit talk to each other through unique connections called synapses. Anomalies that affect communication within neural circuits lead to neurological disorders and affect regular brain functions (Geula, 1998). For over a century, researchers have tried to understand human brain functions using simple animal models such as worms, flies, snails, mice, and rats. Over the past two decades, molecular genetics studies have enabled a common conceptual framework for the development and basic function of the nervous system. However, because many debilitating human disorders are genetically complex and phenotypic screens are difficult to perform, large-scale genomic approaches to discover genes that are uniquely expressed in brain circuits and regions that control complex behaviors are becoming highly significant.

A comprehensive picture of the genomic factors underlying an individual's susceptibility to complex diseases is essential to understanding diseases like PTSD where it is often difficult to make an absolute clinical diagnosis. One such study showed that a single-nucleotide polymorphism (SNP) of the gene Oprl1 is associated with PTSD symptoms in humans and an altered expression of this gene in the amygdala of mouse models led to alterations in fear processing that may lead to PTSD development (Andero et al., 2013). Another study of adult civilians with PTSD has shown that individuals with a history of childhood abuse have very discrete and profound changes in gene activity patterns, compared to adults with PTSD but no history of child abuse (Mehta et al., 2013). The outcome of both these studies provide us the evidence that susceptibility to developing PTSD may be coded in one's DNA; and the exposure to traumatic events may act as the trigger to PTSD onset. In addition, scores of recent studies on epigenetic mechanisms account that it is not just DNA that carries all the information for a specific phenotypic outcome, and specific alterations in epigenetic signatures such as DNA methylation were more frequent in the PTSD group that suffered child abuse (Mehta et al., 2013). The above study also found that the PTSD with child abuse group demonstrated more changes in genes linked with nervous system development and regulation of the immune system, while the PTSD patient group with no child abuse history showed more changes in genes linked with apoptosis (cell death) and regulation of growth rate. Though the symptoms in both the PTSD groups were the same, these findings showed that the specific affected biological pathways might lead to different mechanisms of PTSD symptom formation within the brain. These studies provide ample evidence that PTSD is associated with distinct molecular fingerprints and identifying the key genetic factors for PTSD etiology will not only improve our understanding of the underlying pathophysiology, but may also lead to new avenues for preventing and treating this distressing

disease at an early stage. In recent years, these approaches have been strengthened by the data-driven advancements in science and technology (Duncan et al., 2018; Nievergelt et al., 2018; Sumner, 2017).

46.8 Applications of genomic and transcriptomics methods

Transcriptomics and gene expression profiling methods analyze the expression of thousands of genes simultaneously in a single biological sample by quantifying the levels of individual mRNA transcripts. Some of the earlier methods used for the identification of genes in different parts of brain include differential display, representational difference analysis, serial analysis of gene expression, and massively parallel signature sequencing. These techniques are relatively expensive and laborious, which led to the search for high-throughput screening techniques that can capture dynamic gene expression changes and perform large-scale gene expression profiling in complex neuronal systems.

Microarrays hold the promise of becoming a revolutionary tool for large-scale analyses of genome sequences and gene expression (Noordewier and Warren, 2001; Young, 2000). The most commonly used formats are the oligonucleotide microarray (Fodor et al., 1993) and the cDNA microarray (Schena et al., 1995). Over the years, microarray technology advanced to support maximum coverage of the transcriptome. The successful applications of microarray technology offer a robust and unbiased approach to acquire global gene expression patterns in the whole brain, specific tissue, or a single neuron. cDNA arrays are often used in RNA expression analyses, while oligonucleotide arrays are used for sequence analyses. Oligonucleotide arrays offer a number of advantages over cDNA microarrays such as increased specificity of hybridization, which is key to SNPs (LaForge et al., 2000), mutational analysis (Hacia, 1999), identification of splice forms, and alternatively polyadenylated transcripts (Hu et al., 2001). Microarrays have been extensively used to study expression profiles of complex neuropsychiatric disorders using both animal models (Lin et al., 2012) and RNA from postmortem human brain tissue (Lehrmann et al., 2003). Thus microarrays can be effectively applied toward robust screening of a large number of genes, which are altered with PTSD onset.

Validation of microarray results is often performed by another sensitive technique, such as qualitative real-time PCR (qRT-PCR). qRT-PCR uses fluorescence detection of the PCR product by combining a thermal cycler with a fluorescent spectrophotometer (Higuchi et al., 1993). qRT-PCR reaction can be performed in two different formats. In the first case the double-stranded DNA generated is detected by the binding of a fluorescent dye such as SYBR green I that intercalates only to the double-stranded DNA (Ponchel et al., 2003). The second format uses a fluorescent reporter molecule that is released due to the exonuclease activity of the DNA polymerase enzyme. qRT-PCR is widely used as a robust method for quantitative gene expression analysis (Gibson et al., 1996). Apart from qRT-PCR, another widely used approach to validate gene expression in transcriptomics is in situ hybridization. Fluorescence in situ hybridization (FISH) was developed in the 1980s, and rapidly became a powerful technique (Langer-Safer et al., 1982). Using FISH, a small RNA fragment of the mRNA transcript to be tested is fluorescently labeled and used for hybridization in fixed samples. The probe binds to the complementary sequences within the sense transcript, which can be visualized by fluorescence microscopy. FISH can identify whole chromosomes, centromeres, telomeres, specific regions or genes, or aberrations in interphase tumor nuclei, and can be effectively used to identify novel mRNA transcripts, the levels of specific genes expressed, and their cellular localization. Using this technique, a genome-wide three-dimensional map of the entire human brain is constructed that details where each gene is "turned on or off."

Very often, however, the molecular characterization of clinical samples is complicated and limited by the available amount of samples. Strategies to overcome this problem include amplification of the starting material or of the signal to be detected, or miniaturization of the method. Novel means are also required to measure gene expression with allele-specific and splice-variant-specific profiles. Such technologies promise to be a further big step from bench to bedside. Though microarrays are still widely used, an increasing number of studies now use direct sequencing of transcripts by high-throughput sequencing technologies (RNA-Seq), also known as "next-generation" or "deep" sequencing (Wang et al., 2009). Unlike microarrays, RNA-Seq is not limited to detecting transcripts that correspond to existing genomic sequences and has significantly low, if any, background signal. In addition, RNA-Seq does not have an upper limit for quantification, offering a large dynamic range of expression levels over which transcripts can be detected. Most commonly used techniques to analyze genetic and genomic variants are shown in Fig. 46.1.

SNP genotyping is an advanced genotyping method that measures SNPs between genomes. In an SNP, a single base pair is mutated at a specific locus, leading to malfunction of the specific gene product and a disease phenotype. Over 1 million SNPs are currently reported to be present within the human genome (Sachidanandam et al., 2001). Even though very small numbers of SNPs cause changes in gene function or expression, it is critical

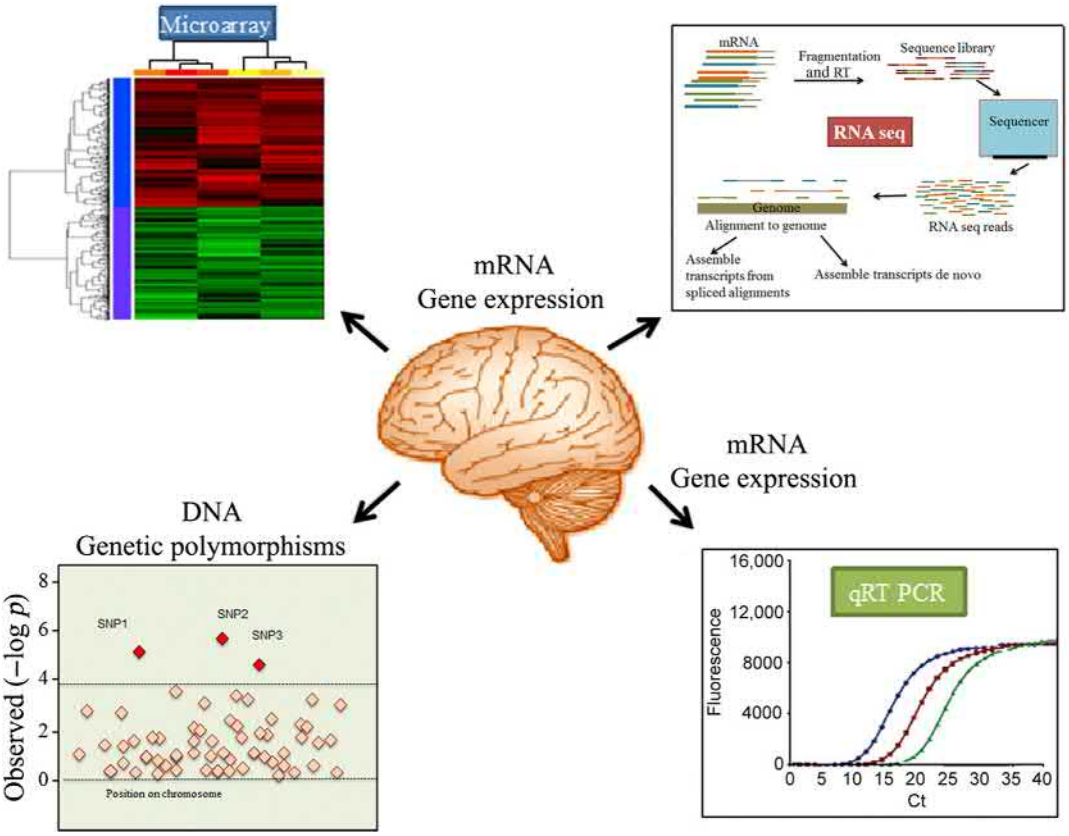

FIGURE 46.1 The most commonly used techniques to identify the genomic and transcriptomic changes associated with specific diseases. In microarray, RNA-seq, and qRT-PCR, mRNA isolated from samples is characterized to observe any changes in gene expression. In single-nucleotide polymorphisms (SNPs), DNA is used to identify a single base pair mutation at a specific *locus* on the chromosome.

to identify SNPs as they are implicated in a number of diseases. There are multiple platforms to perform SNP genotyping and multiple studies have shown specific SNPs are associated with PTSD. A genome-wide SNP association analysis in 1578 European Americans (EAs), 300 of whom suffered from PTSD, and 2766 African Americans, 444 of whom had PTSD using Illumina Omni1-Quad microarray found novel common risk alleles for PTSD (Xie et al., 2013). This study yielded close to 90,000 SNPs and found a new susceptibility gene for PTSD called Tolloid-Like 1 gene. A similar genome-wide association study (GWAS) from a cohort of veterans reported a SNP (rs8042149) located in the retinoid-related orphan receptor alpha gene (RORA) associated with PTSD susceptibility (Logue et al., 2013). RORA has been implicated in prior GWASs of psychiatric disorders and is known to have an important role in neuroprotection and other behaviorally relevant processes. This study represents an important step toward identifying the genetic underpinnings of PTSD.

Another well-studied example is the FK506 binding protein 5 (FKBP5), a glucocorticoid receptor cochaperone regulator (Mehta et al., 2011) that has reduced expression levels in PTSD (Yehuda et al., 2009). In a cross-sectional study on childhood abuse, four specific SNPs of the FKBP5 gene were found to be predictors of adult PTSD symptoms (Binder et al., 2008). These four SNPs were identified as rs3800373, rs9296158, rs1360780, and rs9470080 from a study done on European Americans and African Americans who were originally screened for lifetime PTSD (Xie et al., 2010). All four SNPs showed a similar linking pattern (Shinozaki et al., 2011), which supported a genetic as well as environmental basis for childhood abuse and the subsequent onset of PTSD in adulthood.

In another study the peripheral expression levels of FKBP5 gene expression and volumes of specific brain structures such as the hippocampus, amygdala, and medial orbitofrontal cortex in 39 patients with PTSD were compared before and after cognitive behavioral therapy. Microarray and qRT-PCR analysis have found that there was a significant increase in FKBP5 expression and hippocampal volume in patients with PTSD. Another study in the similar line on Pituitary Adenylate Cyclase—Activating Polypeptide (PACAP)—PAC1 receptor was found to be involved in abnormal stress responses

underlying PTSD in a sex-specific manner in heavily traumatized individuals (Ressler et al., 2011). Analyzing the levels of PACAP in the blood and extensive SNP genotyping of PACAP and PAC1 genes showed a single SNP within PAC1 gene (rs2267735) that predicts PTSD diagnosis and symptoms in females only. The study also found that methylation of the PAC gene in peripheral blood is associated with PTSD.

46.9 Role of noncoding RNAs and epigenetics in posttraumatic stress disorder

Earlier, SNPs associated with a number of genes were described as genomic indicators of PTSD vulnerability. Recent studies provide evidence that epigenetic mechanisms and ncRNAs are also involved in the development of PTSD, increasing the molecular complexity to a further level. An initial animal model study examining DNA methylation of the brain-derived neurotrophic factor (BDNF) gene in rat models of PTSD found a significant increase in BDNF DNA methylation in the dorsal hippocampus, the highest increase in the dorsal CA1 subregion, and a significant methylation decrease in the ventral hippocampus (CA3) following the stress regimen (Roth et al., 2011). Meanwhile, this study found no change in BDNF DNA methylation in the medial PFC or basolateral amygdala. Interestingly, the mRNA levels of BDNF were decreased in both the dorsal and ventral CA1, providing key evidence that traumatic stress can induce CNS gene methylation and alter gene expression in key brain areas leading to the pathophysiology of PTSD.

A recent study on African Americans involved in the Grady trauma project has found that DNA demethylation altered the transcription of FKBP5, leading to long-term dysregulation of the stress hormone system for stress regulation associated with PTSD (Klengel et al., 2013). It has been reported that PTSD patients who faced significant abuse in childhood displayed more changes in gene expression associated with central nervous system development and immune system regulation, whereas those without a history of childhood abuse displayed more changes in gene expression associated with cell death and growth rate regulation. Specific alterations in the DNA methylation profile were up to 12-fold higher in PTSD patients with a history of childhood abuse (Mehta et al., 2013). In a similar study, gene expression profiles in DLPFC Brodmann area of 46 postmortem patients with or without PTSD have been investigated using human mitochondria-focused cDNA microarrays (hMitChip3) (Su et al., 2008). A total of 119 genes were found differentially expressed between the control and PTSD patients and the majority of genes altered in PTSD samples belonged to the neuronal function-survival networks. Similarly, a recent study using bold sera from US military service members evaluated temporal changes in DNA methylation in select promoter regions of immune system-related genes between PTSD diagnosis, pre- and postdiagnosis, and in control patients. This study found reduced mC levels at the promoter regions of a long ncRNA H19 and interleukin-18 (IL18) in those who did not develop PTSD after deployment, while those who did develop PTSD had increased levels of IL18 (Rusiecki et al., 2013).

MicroRNAs (miRNA) have recently emerged as epigenetic modulators of gene expression in psychiatric diseases like schizophrenia and depression (Miller and Wahlestedt, 2010). miRNAs are short, single-stranded RNA sequences that regulate gene expression by binding to the regulatory regions of mRNA and preventing translation, representing another mechanism of regulating gene expression besides up- or downregulation of transcription (Fabian et al., 2010). In a recent study reporting the connection between miRNAs and PTSD, miRNA profiles of the PFCs from fluoxetine-treated and control wildtype C57BL/6N mice were dissected 74 days after they were subjected to either a single traumatic electric footshock or a mock treatment (Schmidt et al., 2013). Fluoxetine is an antidepressant effectively used both in PTSD patients and in mice suffering from a PTSD-like syndrome. Using miRBase 18.0 screening and qPCR validation, the study found five miRNAs, including one (mmu-miR-1971) showing a significant reduction in fluoxetine-treated shocked mice.

The relevance of the noncoding genome to human disease has mainly been studied in the context of miRNA expression and function. However, it is still a start for us to recognize the nature and extent of the involvement of other ncRNAs in disease. A GWAS of PTSD was conducted using a primarily African American sample set of women from the Detroit Neighborhood Health Study that included 94 PTSD cases and 319 controls exposed to at least one traumatic event as well as an independent cohort of primarily European American women from the Nurses Health Study II (NHSII) which was composed of 578 PTSD cases and 1963 controls (Su et al., 2008). More than 700,000 markers were screened using Illumina HumanOmniExpress BeadChip. The results found a genome-wide significant association of one marker mapping to a novel RNA gene, lincRNA AC068718.1. The study also performed pathway analysis to obtain a protein functional interaction network, and found pathways related to telomere maintenance and immune function. This study demonstrated the emerging evidence that ncRNAs may play a crucial role in shaping the landscape of gene regulation with putative pathological effects that lead to phenotypic differences.

46.10 Toxic chemical exposure and human diseases

Scores of studies point to the influence of an individual's genetic information and social conditions in the development of PTSD. However, it is also important to address the role of other external factors such as brain cell damage and nervous system damage caused by chemical exposure. It has been reported that residents from regions of high-intensity warfare and chemical weapons during the 1980–88 Iran–Iraq War significantly had higher risks of meeting criteria for lifetime and current PTSD compared with the residents of high-intensity warfare alone (Hashemian et al., 2006). Currently, PTSD development following trauma or exposure to chemical war agents (CWAs) is poorly understood. Although every individual with PTSD has been exposed to a traumatic event, studies show that the majority of people who experience trauma do not develop PTSD. It is intriguing why some people remain resilient; some experience short-term difficulties; and others develop a chronic problem such as PTSD.

According to the Research Advisory Committee report on "Scientific Progress in Understanding Gulf War Veterans' Illnesses: Report and Recommendations," almost 30% of the veterans from the Gulf War were disabled by chronic symptoms such as severe headaches, memory problems, confusion, dizziness, blurred vision, and tremors (http://www.va.gov/RAC-GWVI/Gulf_War_Illnesses_links.asp). Notably, Gulf War veterans have developed ALS at twice the rate of veterans who did not serve in the Gulf War. Based on the research data collected, the committee identified the nerve gases sarin and pyridostigmine bromide, and multiple pesticides as potential factors in the development of neurological disorders. More research is needed to better understand the veterans' vulnerability to PTSD after exposure to CWAs, especially to consider why only a small portion of the veterans who experienced childhood abuse developed the symptoms.

46.11 Genomic applications: understanding the relationship between posttraumatic stress disorder and chemical toxicity

The development of modern genomic analysis techniques offers an unparalleled opportunity to investigate neurological diseases such as PTSD due to CWA and other toxic chemical exposure. CWAs like organophosphate (OP) insecticides and nerve gases are capable of changing neuronal activity primarily via cholinergic pathways. These CWAs bind irreversibly to acetylcholinesterase (AChE), an important regulator of the neurotransmitter acetylcholine (ACh) that leads to lasting changes in neuronal activity. Studies in rodent brains have shown that the exposure could affect brain regions critical for attention, anxiety, and addiction. Nerve agents affect central and peripheral nervous systems, and cardiovascular, respiratory, gastrointestinal, and metabolic systems. Most importantly, they cause behavioral and psychological changes in humans, leading to memory loss and depression. One of the nerve agents used in previous wars, sarin (GB), is suspected to be one of the key factors responsible for Gulf war syndrome.

Animal models have greatly aided our understanding of the effects of CWAs on the cellular functions, molecular pathways, organ functions, and gene expression changes that are conserved in humans. Earlier studies on rats showed that sarin usually induces neurotoxicity by affecting the mRNA expression of alpha tubulin in the CNS. The study used northern blot to look at the differential expression of alpha tubulin mRNA in different regions of rat brain. Another similar study found that there is a differential distribution of AChE mRNA expression following exposure to sarin (Damodaran, 2009). These studies clearly showed that exposure to sarin causes gene expression changes in the CNS that may lead to neurotoxicity as well as behavioral and psychological changes. Another study looked at sarin-induced global gene expression pattern changes at different time points after exposure by microarray (Damodaran et al., 2006; Damodaran, 2009). Analysis of gene expressions at an early time point (15 min; $0.5 \times LD_{50}$) and a later time point (3 months; $1 \times LD_{50}$) identified specific gene expression changes to each time point and seven genes that were consistently altered in both time points (Ania-9, Arrb-1, CX-3C, Gabab-1d, Nos-2a, Nrxn-1b, and PDE2). Further genome-wide study in the rat brain at another early time point (2 h: $0.5 \times LD_{50}$) following sarin exposure showed 46 genes were significantly altered in comparison to the control animals. Most of these 46 genes belong to ion channels, calcium channels, and binding protein families. The study also found many genes are involved in the pathogenesis of sarin-induced pathology and OP-induced delayed neurotoxicity. These studies indicate that exposure to sarin can lead to neurodegeneration at a later time and result in neuropathological alterations. Pachiappan and colleagues applied single (3 and 24 h) or repeated (2×24 h) doses of sarin (5 μg/mL) on human neuronal cells (SH-SY5Y) to identify altered gene expressions (Pachiappan et al., 2009). Microarray analysis identified over 200 genes that were significantly altered following sarin administration in this study. The study also found repeated doses over 48 h persistently downregulated genes linked to neurodegenerative mechanisms indicating the adverse effect of sarin exposure for prolonged time.

O-Ethyl-*S*-2-diisopropylaminoethyl methylphosphonothiolate (VX), another member of the OP compounds family, used in chemical warfare, is a very potent nerve agent. A recent microarray study using cultured human neural cells (hN2) exposed to 0.1 or 10 μM of VX for 1 h has reported changes in gene expressions after 6, 24, and 72 h (Gao et al., 2013). The altered genes were subjected to functional pathway analysis and many of them were found to be involved in pathways related to nervous system development and function. Advancement in genomics may also shed light on some other poorly understood toxic effects of nerve agents. It has been reported that civilians exposed to industrial OPs, such as pesticides, have a higher incidence of Parkinson's disease (Hatcher et al., 2008; Manthripragada et al., 2010), though it is not clear whether nerve agents lead to similar neurodegenerative effects. There are many relevant questions in conjunction with neurological disorders and the use of CWAs. Some questions to be asked are whether long-term regional differences seen in the brain after nerve agent exposure also reflected in large-scale gene expression changes. Can we correlate changes in gene expression profiles with altered behavioral patterns? And does neuroinflammation, one of the acute effects of nerve agents (Chapman et al., 2006; Svensson et al., 2001; Williams et al., 2003) diminish across time? Genomics analysis of subjects exposed to CWAs may provide us ample information to answer these questions and provide additional information as we approach an understanding of the spectrum of chronic effects of nerve agent exposure.

Microarrays are still highly useful to characterize changes in gene expression due to a toxicant of interest, identify up- or downregulated genes, map regulatory pathways modulated by the toxicant, and, in the case of CWAs, identify potential therapeutic targets in these pathways (Hamadeh et al., 2002; Thomas et al., 2001). Nevertheless, RNA-seq offers high technical reproducibility and large dynamic range compared to microarray, and can be used effectively to identify differential gene expression to understand complex disorders like PTSD. Recent efforts such as the consortium called STRONG STAR Consortium to Alleviate PTSD (STRONG STAR-CAP) led by the University of Texas Health Science Center at San Antonio are employing next-generation sequencing and mass spectrometry to research PTSD and its treatment. The enormous potential of RNA-Seq offers an invaluable resource to investigate the transcriptomic profile of the brain in different layers. A similar recent study showed a transcriptomic analysis of distinct regions of the Alzheimer's disease (AD) brain using Illumina RNA-Seq analysis to examine gene expression levels, splicing isoforms, and alternative transcript start sites from the total brain, frontal, and temporal lobe of healthy and AD postmortem tissue, which found a significant representation of genes associated with neuronal cytological structure and synapse function.

46.12 Proteomics

High-throughput genomic and transcriptomics approaches are indispensable to understanding the molecular map of complex diseases. However, their application is limited by the fact that they can only assess gene expression changes. Translation of mRNA into protein is a highly regulated and complex mechanism and modulated at different levels. Effects due to changes in protein expression, modification, or function can lead to development of diseases and only be inferred from analysis of protein expression profiling. The proteome was initially defined as the complete complement of proteins that are expressed by a genome, then it was denoted as "the total set of expressed proteins by a cell, tissue or organism at a given time under a determined condition" by Wilkins et al. (1996). To assess protein expression status, different proteomics techniques need to be utilized.

Proteomics is widely applied in biological sciences to study protein expression, posttranslational modification, protein−protein interactions, and protein−nucleic acid interactions. It gives us a new platform for studying complex biological functions involving large numbers and networks of proteins (Husi and Grant, 2001; Martins-de-Souza et al., 2011). Since Patrick O'Farrell first presented two-dimensional gel electrophoresis (2DE) in 1975, proteomics has given rise to a new scientific approach for comparative global proteome analyses, especially when combined with mass spectrometry (MS) for protein identification. As a result of the development of in-gel digestion protocols and related optimizations (Shevchenko et al., 1996), the resolution power of 2DE can lead to the separation of more than 2000 protein spots on large-format gels. So far 2DE, followed by MS, is the most used proteomic technique in studies of psychiatric disorders (Ditzen et al., 2006; Kromer et al., 2005). Typically, mass spectrometers have three main features: an ionization source (e.g., matrix-assisted laser desorption/ionization and electrospray ionization), a mass analyzer (e.g., time of flight and quadrupole) and a detector (Bayes and Grant, 2009), that measures the mass-to-charge (m/z) ratio of ionized particles. The 2DE-MS approach provides direct information on intact proteins and protein isoforms. Fig. 46.2 depicts the main techniques currently used in proteomics methodology for PTSD diagnosis.

Link et al. (1999) launched shotgun proteomics in 1999, which increased the capacity for proteome characterization. Shotgun-MS was originally designed as a

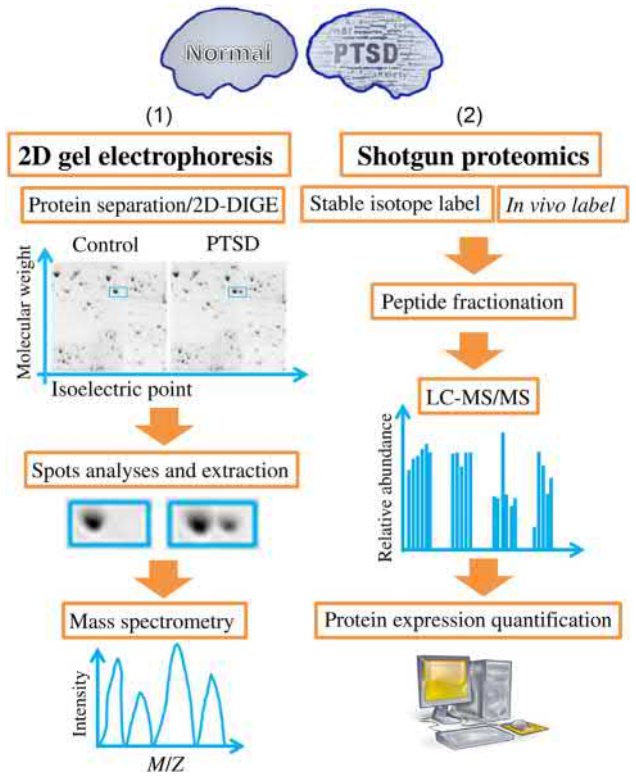

FIGURE 46.2 Major techniques used in proteomics methodology for PTSD diagnosis: (1) two-dimensional gel electrophoresis for proteomics, 2D-DIGE: proteomes mixed in gel; (2) shotgun proteomics methodology.

nongel/MS-direct approach, which was regarded as a more sensitive and reproducible proteome representation compared with 2DE proteomics. Shotgun-MS includes shotgun-MS, liquid chromatography-tandem MS (LC-MS/MS), and multidimensional protein identification technology (MudPIT). Basically, this approach involves digesting the whole proteome of interest using specific enzymes and subsequently identifying the resulting peptides by MS, while proteome quantification and sample comparison rely on a label-free MS methodology or stable isotope labeling. Mass spectrometric (MS) analysis is capable of identifying small modifications, like point mutations, phosphorylation, and glycosylation; it can also detect the primary structure of proteins and peptides, for example, amino acid sequences (Davidsson et al., 2003). Based on its applications, proteomics includes (1) expression proteomics, which studies protein expression profiles; (2) comparative proteomics, which compares physiological and diseased states; (3) Structural proteomics for structure investigations; and (4) functional proteomics, which studies the interactions between molecules. Due to the high levels of accuracy and ultrasensitivity features, proteomics has become a preeminent tool in many areas, especially in neuroscience (Tyers and Mann, 2003).

46.13 Neuroproteomics: proteomics applications in neuroscience

"Neuroproteomics" is the study of the proteomes of the nervous system (Bayes and Grant, 2009) and is of great importance in functional studies. Bayes and Grant (2009) have reviewed four major categories of neuroproteomics, including: (1) expression neuroproteomics, which refers to the qualitative and quantitative cataloging or profiling of neuroproteome; (2) functional neuroproteomics, which addresses functional properties of individual proteins as well as their organization into substructures, complexes, and networks; (3) clinical neuroproteomics, which includes the identification of biomarkers and disease mechanisms for neurological, neurodegenerative, and psychiatric diseases which also benefit drug discovery; and (4) neuroproteomic informatics, which handles analyzing proteomic data sets using computational tools and databases. A typical example of a proteomic approach to study the effect of complex neurological diseases is the shotgun analysis of *postmortem* dorsolateral PFC brain tissue from major depression disorder (MDD) patients (Martins-de-Souza et al., 2012). Gel electrophoresis followed by shotgun data-independent label-free liquid chromatography-mass spectrometry led to identification of distinct proteome fingerprints between MDD and control subjects. Another study to tackle the proteomic profile of anxiety disorder applied a quantitative proteomic approach where metabolic labeling of an HAB/LAB mouse model with stable isotopes was used to identify a large number of proteins in a high-throughput manner (Zhang et al., 2011). Differentially expressed proteins were subjected to pathway discovery analyses that suggested several biological processes and pathways to be affected in the genetic predisposition to extremes in trait anxiety. Though these studies did not draw any connection between the proteins and pathways connected with PTSD, they may trail blaze similar efforts toward proteomic research.

46.14 Proteomics approaches to understand natural and chemical toxicity-induced posttraumatic stress disorder

Despite the enormous medical and economic consequences of traumatic injury to the CNS, relatively little work has been directed toward elucidating the proteomic profile of posttraumatic psychopathology due to the complexity of the molecular mechanisms of the disease. Some of these proteins may serve as diagnostic and prognostic markers to assess the severity of tissue damage. Studies of proteomic biomarkers of PTSD risk have been in play

for a long time; yet they are limited by many technical issues such as optimizing sample collection time frame and the variation in the timing between the traumatic incident and the biological sample collection. Despite these limitations, the initial research provided key insights into the biological mechanisms underlying PTSD vulnerability. A key observation from initial proteomics studies was the high concentrations of inflammatory proteins and, together, these studies indicated increased concentrations of inflammatory cytokines related to the risk for PTSD onset (Gill et al., 2008, 2010). Proteomics makes it possible to identify molecular mechanisms and molecular targets for PTSD through large-scale screening of patients and a validated animal model for protein expression profile analysis. In a recent study, large-scale proteomic approach and mass spectrometry with the use of human proteomic databases were employed to identify the differential serum proteomic profile in combat-related PTSD subjects and healthy controls (Dragica Kozaric-Kovačic et al., 2010). The study found more than 100 proteins expressed differently between individuals with PTSD and healthy controls. These results indicated the direction for a larger analysis of this type in people with PTSD. This pilot study offers a good basis for further proteomic research, which could help in better diagnosis and treatment of PTSD, as well as clarification of its etiology.

Sulfur mustard [SM, bis-(2-chloroethyl) sulfide], a potent alkylating agent and one of the chemical reagents used in chemical warfare, is indicated in PTSD development. A quantitative proteomic approach using stable isotope labeling combined with immobilized metal affinity chromatography tested the effect of SM in a human keratinocyte cell culture model. The study found large-scale protein phosphorylation changes resulting from SM exposure and characterized over 2300 phosphorylation sites, many of which showed altered levels in response to SM treatment. The study also found new proteins that are associated with SM toxicity (Everley and Dillman, 2010). Global organizations, including governmental agencies, have taken an interest and invested in finding data-driven solutions in the field (Koenen, 2019). Further, there is an enormous effort from the pharmaceutical industry to find new drugs to relieve pain and eventually to cure such a complex disease (Melville, 2017).

46.15 Concluding remarks and future directions

It is clear that PTSD is not a monolithic simple disorder, which can be characterized by unique and consistent mental and biological traits, as the development of this disorder results from complex interactions among numerous factors. Genetic background as well as environmental factors may contribute to whether one is sensitive to complex circumstances such as trauma exposure, leading to PTSD development. Recent research studies provided solid evidence that specific DNA functions can be modified by such exposure through epigenetic pathways, resulting in alterations in gene expression leading to the pathological phenotype. In this chapter, we have provided a comprehensive picture of the technological advances in genotyping, expression profiling, and proteomics that offer exciting and promising advances in understanding the basis of PTSD as well as improved diagnosis and therapy. Additionally, short- and long-term exposure to toxic chemicals, such as OP nerve agents and insecticides, and other chemical warfare agents contribute toward PTSD onset. The combination of genomic and proteomic information will allow early and more accurate prediction of individuals' susceptibility to chemical exposure-related trauma, development of PTSD, and disease progression. Though significant discoveries have been made, there is clearly substantial promise and potential remaining to be fully realized through increasing the use of and further development of -omic technologies. Governmental organizations and the pharmaceutical industry have taken an enormous interest in accelerating effort in this field to enable a difference to be made in that "waiting" PTSD patient, sooner.

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Chapter 47

Excitotoxicity, oxidative stress, and neuronal injury

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47.1 Introduction

Exposure to anticholinesterase (anti-AChE) agents, organophosphates (OPs), and carbamates (CMs) in the form of insecticides and chemical warfare agents (CWAs) affects or threatens much of the world's population. The widespread use and easy accessibility to more than 100 different OPs used as insecticides worldwide result in a huge number of intoxications and several hundred thousand fatalities annually (Gunnell and Eddleston, 2003). Other derivatives of phosphoric acid, nerve agents used in warfare, are considered the most toxic compounds of all chemical weapons. The devastating effects of these agents were demonstrated during the Iraqi conflict with Iranian troops and Kurdish civilians, as well as a terrorist attack on the Tokyo subway train system that occurred in 1995, resulting in over 5500 casualties (Nozaki et al., 1995; Nagao et al., 1997). Terrorist attacks involving warfare nerve agents, weapons of mass destruction, or other industrial chemicals present worldwide security threats and health concerns. Therefore, anti-AChE agents represent a significant potential threat not only to the military, but also to the general civilian population.

Pharmacologically, all these compounds are acetylcholinesterase (AChE) inhibitors. Their acute symptoms are attributed to accumulation of acetylcholine (ACh), thus exhibiting cholinergic toxicity. Phosphorylation of the esteratic site of the enzyme diminishes its capacity to catalyze endogenous substrate ACh (Taylor et al., 1990). Most OP compounds combine with AChE only at the esteratic sites, and the stability of the bond depends upon the structure of the compound that is attached. OP compounds containing larger alkyl groups may hinder cleavage, leaving the phosphorylated AChE inactivated almost indefinitely. As a result, normal activity recurs only upon the synthesis of a new enzyme. This process is known as *aging*, and its time course varies depending on the nerve agent. Consequently, the hydrolysis of ACh is prevented, leading to accumulation of ACh in the synaptic cleft and overstimulation, followed by the desensitization of muscarinic and nicotinic ACh receptors.

The constantly activated nicotinic cholinergic receptors generate involuntary skeletal muscle contraction, followed by complete depolarization block, the clinical manifestation of which is flaccid paralysis. In a manner similar to the events in the peripheral nervous system (PNS), the accumulation of ACh in central nervous system (CNS) nerve endings causes anxiety, disorientation, and general convulsions, followed by loss of consciousness and respiratory arrest. Anti-AChE agent-induced ACh accumulation at the muscarinic sites also enhances the activity of various secretory glands, leading to excessive salivation, lacrimation, bronchorrhea, diarrhea, and sweating. The severity of poisoning by nerve agents varies from minor cases (mild dyspnea, blurred vision, and glandular hypersecretion) to more severe poisoning, which is characterized by severe dyspnea, skeletal muscle fasciculation, convulsions, and unconsciousness, which occurs soon after an intense exposure of only a few minutes (Goldfrank et al., 1982; Weinbroum, 2005). Thus, depending upon the degree of AChE inhibition, cholinergic stimulation may also lead to respiratory failure, coma, and death.

Convulsions are a major sign of OP nerve agent poisoning (Misulis et al., 1987). OP-induced seizures rapidly progress to status epilepticus (SE), which leads to profound structural brain damage (Lemercier et al., 1983; McLeod, 1985). Excitotoxic levels of glutamate following soman exposure are thought to be involved in the

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dendritic and synaptic damage as an early toxicological response that leads to neuronal dysfunction and memory impairment (Carpentier et al., 1991). Anti-AChE exposure is also associated with oxidative stress, synaptic architecture dysfunction, and cellular deterioration in the brain, especially in the hippocampus (Johnson et al., 2008; Zaja-Milatovic et al., 2009; Milatovic et al., 2010).

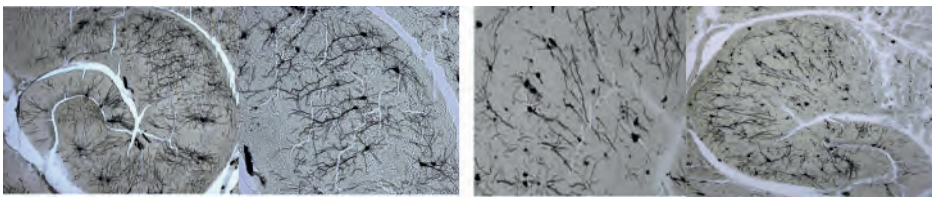
47.2 Excitotoxicity and oxidative injury

The most consistent pathological findings in acute experiments with anti-AChE agents include degeneration and cell death in the pyriform cortex, amygdala, hippocampus (where the CA1 region is preferentially damaged), dorsal thalamus, and cerebral cortex. It has been shown that soman-induced seizures produce an increase in extracellular glutamate in the pyriform cortex (Wade et al., 1987) and the cornu ammonis (CA) region of the hippocampus (Lallement et al., 1992), followed by activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors in the CA1 region. Moreover, glutamate stimulates the continuous release of ACh (Anderson et al., 1994), contributing to further excitatory stimulation, prolongation of the seizures, and neurodegeneration in vulnerable brain regions (Wade et al., 1987; Lallement et al., 1991, 1992). This excitotoxic injury caused by increased levels of glutamate also causes cognitive dysfunction (Phillips et al., 1998; O'Dell et al., 2000; Faden et al., 2001). Increased synaptic glutamate concentrations following OP exposure also alter glutamate receptor expression (Piehl et al., 1995; Cebers et al., 2001) and perturb NMDA receptor subunit distribution, thus changing the overall physiology of the receptor and the functionality of the hippocampus (Cebers et al., 1999).

Although seizures can induce neuronal death, they may also have nonlethal pathophysiological effects on neuronal structure and function. Dendritic spines represent the structural sites of contact for the majority of excitatory, glutamatergic synaptic inputs into neurons, and they are strongly implicated in mechanisms of synaptic plasticity and learning. NMDA and other glutamate receptor subtypes are clustered in dendritic spines (Rao and Craig, 1997; O'Brien et al., 1998), which serve as integrative units in synaptic circuitry and participate in synaptic plasticity (Yuste and Denk, 1995). The accumulation of glutamate receptor clusters in spines is governed by excitatory synaptic activity and increases when activity is suppressed (Rao and Craig, 1997; O'Brien et al., 1998). Conversely, excitotoxicity produces a rapid and profound loss of dendritic spines in cultured neurons (Halpain et al., 1998), mimicking the loss in dendritic spine synapses in neurological conditions, including epilepsy, aging, and schizophrenia (Jeffrey et al., 1997; Jiang et al., 1998). This suggests that receptor localization at synapses might be critical to excitotoxicity and govern neuronal vulnerability to excitotoxicity.

Earlier studies investigating the role of glutamate receptors in mediating seizure-induced brain damage showed that kainic acid (KA)-induced epilepsy damaged limbic structures in rats (Ben-Ari et al., 1980). Kainate is a rigid analog of glutamate, the principal excitatory neurotransmitter in the CNS, and it is a very potent stimulant of a subset of the ligand-gated ion channel, called KA receptors (Milatovic et al., 2005b). Activation of the KA subtype of ionotropic glutamate receptors results in sustained epileptic activity in the hippocampus, followed by a selective pattern of neuropathology that is similar to human temporal lobe epilepsy (Schwob et al., 1980; Ben-Ari and Cossart, 2000). Kainate administration and intense seizure activity associated with SE are sufficient to induce oxidative stress, degeneration of hippocampal CA neurons, and hyperexcitability of surviving hippocampal CA neurons (Ben-Ari, 2001; Dong et al., 2003; Zaja-Milatovic et al., 2008) (Fig. 47.1).

The hypothesis concerning OP-induced neuronal oxidative injury is that overstimulation of glutamatergic receptors results in sustained epileptic activity in the hippocampus and neuropathologic changes predominantly in the pyramidal neurons. Cell damage is thought to result from intense transient influx of calcium, leading to mitochondrial functional impairment characterized by activation of the permeability transition pores in the inner mitochondrial membrane, cytochrome c-release, depletion of adenosine triphosphate (ATP), and simultaneous formation of reactive oxygen species (ROS)



Saline exposure Kainic acid exposure

FIGURE 47.1 Photomicrographs of mouse hippocampi with pyramidal neurons from the CA1 hippocampal area of brains 1 h after saline (control) and kainic acid (KA, 1 nmol/5 μL, ICV) injections. Treatment with KA induced degeneration of the hippocampal dendritic system and a decrease in the total length of the dendrite and spine density of hippocampal pyramidal neurons.

(Cadenas and Davies, 2000; Patel, 2002; Nicholls et al., 2003). In addition, an increase in cytoplasmic calcium ions triggers intracellular cascades through stimulation of enzymes, including proteases, phospholipase A_2, and nitric oxide synthase (NOS), which also leads to increased levels of free radical species and oxidative stress (Lafon-Cazal et al., 1993; Farooqui et al., 2001). Since free radicals are direct inhibitors of the mitochondrial respiratory chain, ROS generation perpetuates a reinforcing cycle, leading to extensive lipid peroxidation and oxidative cell damage (Cadenas and Davies, 2000; Cock et al., 2002). Previous studies have supported a role of oxidative stress and excessive generation of ROS and reactive nitrogen species (RNS) in anti-AChE-induced neurotoxicity (Dettbarn et al., 2001; Gupta et al., 2001a,b, 2007; Milatovic et al., 2005a; Zaja-Milatovic et al., 2009).

Two radicals that play predominant roles as initiators of lipid peroxidation are the hydroxyl radical (OH^-) and the peroxynitrite radical ($OONO^-$). The superoxide anion radical (O_2^-), which is generated during the electron transport process in mitochondria, is involved in the generation of both OH^- and $OONO^-$. Superoxide dismutase (MnSOD and Cu/ZnSOD) converts O_2 to hydrogen peroxide (H_2O_2), which is then converted to OH^- via the Fenton reaction, catalyzed by Fe^{2+}, Cu^+, or Mn^{2+}. $OONO^-$ is generated from the interaction of NO with O_2^-. A major stimulus for NO production is the elevation of intracellular Ca^{2+}, which binds to calmodulin, resulting in the activation of NOS. Peroxynitrite is a powerful oxidant exhibiting a wide array of tissue-damaging effects, including lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration, and inhibition of mitochondrial respiration (Virag et al., 2003). Peroxynitrite, which dissipates during oxidation (Wang et al., 2003), has also been found to induce nitration as well as oxidation of adenine, guanine, and xantine nucleosides (Sodum and Fiala, 2001). Low concentrations of peroxynitrite trigger apoptotic death, whereas higher concentrations induce necrosis with cellular energetics [ATP and nicotinamide adenine dinucleotide (NAD)] serving as a switch between the models of cell death. ROS and RNS produced under oxidative stress are known to damage cellular biomolecules including lipids, sugars, proteins, and polynucleotides and to initiate detrimental effects (Negre-Salvayre et al., 2010; Roberts et al., 2010; Marrocco et al., 2017; Cheignon et al., 2018). Together, ROS- and RNS-damaged biomolecules may induce a variety of cellular responses through the generation of secondary metabolic reactive species (Fig. 47.2).

Thus, increased ROS and RNS production has been directly linked to oxidation of proteins, deoxyribonucleic acid (DNA), and lipids, which may cause injury or induce a variety of cellular responses through the generation of secondary metabolic reactive species (Fig. 47.2).

47.3 Lipid peroxidation and in vivo markers of oxidative damage

Due to a high concentration of substrate polyunsaturated fatty acids (PUFAs) in cells, lipid peroxidation is a major outcome of free radical-mediated injury (Montine et al., 2002a,b). A critical aspect of lipid peroxidation is that it will continue until the oxidizable substrate is consumed or termination occurs, making it fundamentally different from many other forms of free radical injury, in that the self-sustaining nature of the process may entail extensive tissue damage (Porter et al., 1995). Decreased membrane fluidity following lipid peroxidation makes it easier for phospholipids to exchange between the two halves of the bilayer, increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels (e.g., K^+ and Ca^{2+}), and damage membrane proteins, inactivating receptors, enzymes, and ion channels (Halliwell and Gutteridge, 2006; Halliwell, 2007). Increases in Ca^{2+} induced by oxidative stress can activate phospholipase A_2, which releases arachidonic acid (AA) from membrane phospholipids. The free AA can then both undergo lipid peroxidation (Farooqui et al., 2001) and act as a substrate for eicosanoid synthesis (Milatovic et al., 2011a). Increased prostaglandin (PG) synthesis is immediately linked to lipid peroxidation because low levels of peroxides accelerate cyclooxygenase (COX) action on PUFAs (Smith, 2005). Phospholipase A_2 can also cleave oxidized AA residue from membranes.

The use of reactive products of lipid peroxidation as in vivo biomarkers is limited because of their chemical instability and rapid and extensive metabolism (Gutteridge and Halliwell, 1990; Moore and Roberts, 1998). For these reasons, other more stable lipid products of oxidative damage have generated intense interest in recent years as in vivo markers of oxidative damage. These compounds include the F_2-isoprostanes (F_2-IsoPs), F_4-neuroprostanes (F_4-NeuroPs), and isofurans (IsoFs) (Morrow et al., 1990; Fessel et al., 2002; Milatovic and Aschner, 2009; Janicka et al., 2010; Milatovic et al., 2011b; Milne, 2017; Van't Erve et al., 2017).

F_2-IsoPs are PG-like compounds that are produced by a non-COX free radical-catalyzed mechanism involving the peroxidation of AA. Formation of these compounds initially involves the generation of four positional peroxyl radical isomers of arachidonate, which undergo endocyclization to PGE_2-like compounds. These intermediates are reduced to form four F_2-IsoP regioisomers, each of which can consist of eight racemic diastereomers (Morrow et al., 1990; Milne et al., 2015). In contrast to COX-derived PGs, nonenzymatic generation of F_2-IsoPs favors the formation of compounds in which the stereochemistry of the side chains is oriented *cis* in relation to

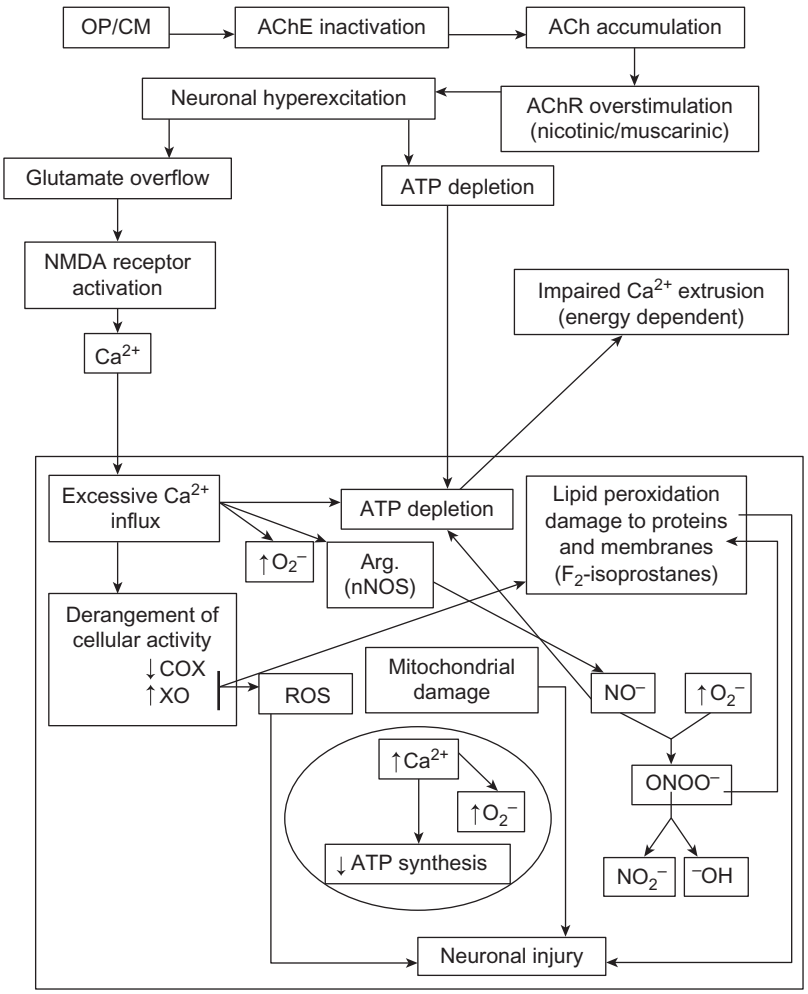


FIGURE 47.2 A schematic diagram showing possible mechanisms involved in an anti-AChE-induced neuronal injury or death by excessive production of ROS/RNS, leading to lipid peroxidation.

the prostane ring. A second important difference between F_2-IsoPs and PGs is that F_2-IsoPs are formed primarily in situ, esterified to phospholipids, and subsequently released by phospholipases (Gao et al., 2006), whereas PGs are generated only from free AA (Morrow et al., 1990; Milatovic et al., 2011a; Milne et al., 2015).

The measurement of F_2-IsoPs is a method that has been extensively replicated as an efficient means of quantifying free radical damage in in vivo models associated with neurodegenerative diseases, including Alzheimer's disease (Montine et al., 1999; Milne et al., 2015), inflammation (Milatovic et al., 2003), metal toxicity (Milatovic et al., 2009, 2011c), excitotoxicity (Milatovic et al., 2005b; Zaja-Milatovic et al., 2008, 2009), cancer, and genetic disorders (Milne et al., 2015). Since AAs present throughout the brain and in different cells in the brain at roughly equal concentrations, F_2-IsoPs reflects damage to brain tissue, but not necessarily to neurons.

Similar studies of lipid peroxidation products have been performed for other substrate lipids. Of particular interest are oxidation products of docosahexaenoic acid (DHA), which have been termed F_4-NeuroPs (Roberts et al., 1998). In contrast to AA, which is evenly distributed in all cell types in all tissues, DHA is highly concentrated in neuronal membranes (Salem et al., 1986; Montine et al., 2004). Thus, determination of F_4-NeuroPs permits the specific quantification of oxidative damage to neuronal membranes in vivo (Montine et al., 2004; Milatovic and Aschner, 2009). In fact, to our knowledge, F_4-NeuroPs are the only quantitative in vivo marker of oxidative damage that is selective for neurons.

Another F_2-IsoPs analog may be formed by peroxidation of eicosapentaenoic acid (EPA, C20:5, $\omega-3$) that leads to the production of F_3-IsoPs. Levels of F_3-IsoPs can significantly exceed those of F_2-IsoPs generated from AA, perhaps because EPA contains more double bonds and is therefore more easily oxidizable (Gao et al., 2006). It has also been shown that in the presence of increased oxygen tension in the microenvironment in which lipid peroxidation occurs, an additional oxygen insertion step

may take place (Fessel et al., 2002; Milatovic and Aschner, 2009). This step diverts the IsoP pathway to form tetrahydrofuran ring-containing compounds termed IsoFs, which are functional markers of lipid peroxidation under conditions of increased oxygen tension. (Milne et al., 2015). Thus, measurements of IsoFs represent a much more robust indicator of hyperoxia-induced lung injury than measurements of F_2-IsoPs. Like IsoPs, IsoFs are chemically and metabolically stable, so they are well suited to act as in vivo biomarkers of oxidative damage (Milne et al., 2015; Van't Erve et al., 2017).

The enzymatic and free radical peroxidation of PUFAs which contains at least three double bonds, like AA and DHA, could lead to malondialdehyde (MDA). This product can be generated by thromboxane synthase, but a report from the Biomarkers of Oxidative Stress Study (BOSS) showed that peripheral levels of MDA derive primarily from nonenzymatic peroxidative degradation of unsaturated lipids (Kadiiska et al., 2005). MDA-TBA adducts, produced in reaction of MDA with thiobarbituric acid (TBA) are used to spectrophotometrically measure the levels of oxidative stress and consequent lipid peroxidation (Spickett et al., 2010; Fang et al., 2017). 4-Hydroxy-2-nonenal (HNE) is also a reactive aldehyde arising from peroxidation of ω6 fatty acid (Uchida, 2003; Guéraud et al., 2010; Barrera et al., 2018). HNE is formed under various conditions like auto-oxidation and stimulated microsomal lipid peroxidation (Neely et al., 2005). MDA and HNE can covalently modify proteins and alter their functions (Butterfield et al., 2006). In addition to protein modification, these lipid peroxidation products can interfere with the synthesis of DNA and ribonucleic acid (RNA), alter cell metabolism and signaling, and mediate brain-induced oxidative damage. Several studies suggest that MDA and HNE can promote the degeneration of cholinergic neurons, Aβ aggregation, and amyloidogenesis (Pedersen et al., 1999; Butterfield et al., 2006).

ROS and RON can react with the DNA molecule and induce purine or pyrimidine base or sugar lesions, nitration and deaminations of purines, and DNA−DNA or DNA−protein cross-links (Dizdaroglu et al., 2002). These processes lead to mutations and impaired transcriptional and posttranscriptional processes and compromise protein synthesis (Colurso et al., 2003). The most investigated DNA adduct, 8-hydroxy-2′-deoxyguanosine (8-OHdG), can be evaluated by multiple techniques including GC-MS, HPLC, LC-MS, immunoassay, and capillary electrophoresis (Lovell and Markesbery, 2007; Fang et al., 2017).

In addition, DNA damage, oxidative phosphorylation, and altered cell metabolism may lead to apoptosis and promote neuronal death (Fishel et al., 2007; Becker and Bonni, 2004). ROS and RNS can also attack amino acids, leading to the protein oxidation and formation of carbonyl derivatives (Stadtman and Levine, 2003; Davies, 2016). Oxidation of protein also leads to protein fragmentation and protein cross-linking. In addition, peroxynitrite and a hydroxyl radical can react with tyrosine and form other indexes of protein oxidation, 3-nitrotyrosine and ortho-tyrosine, respectively. These protein products are relatively stable, with sensitive assays available for their detections (Chakravarti and Chakravarti, 2007; Davies, 2016).

47.4 Anti-AChE-induced seizures, oxidative injury, and neurodegeneration

Lipid peroxidation, mitochondrial dysfunction, reduced neuronal energy levels, and reduced cytochrome c-oxidase (COx) activity support the contention that anti-AChEs, such as diisopropylfluorophosphate (DFP) and carbofuran (CF), cause neuronal injury by excessive formation of ROS (Yang and Dettbarn, 1998; Milatovic et al., 2000a,b, 2001, 2005a; Gupta et al., 2001a,b). Additionally, our studies showed that seizure-induced cerebral oxidative damage in adult animals is accompanied by alterations in integrity of the hippocampal CA1 dendritic system (Gupta et al., 2007; Zaja-Milatovic et al., 2008, 2009; Milatovic et al., 2010).

A single injection of DFP [1.5 mg/kg, subcutaneously (s.c.)] or another AChE inhibitor, CF (1.5 mg/kg, s.c.), produces toxic signs in rats, including salivation, tremors, "wet dog" shakes, fasciculations, and mild to moderate seizures with rearing and rolling over, with progression to severe seizures within 7−15 min (Milatovic et al., 2006; Gupta et al., 2007; Zaja-Milatovic et al., 2009). Signs of maximal intensity such as severe muscle fasciculations, seizures, and convulsions develop within 15−30 min and last for more than 2 h before tapering off. By 24 h, animals are free of toxic signs. The observed signs are typical of anti-AChE toxicity and reveal the involvement of both the CNS and the PNS (Gupta et al., 2001a,b; Milatovic et al., 2005a). Analysis of brains from saline-treated control rats revealed regional variability in brain AChE activity (cortex, 222.0 ± 10.7; amygdala, 529.2 ± 10.29; and hippocampus 301.2 ± 9.5 μmol/g wet weight). A single acute dose of DFP (1.5 mg/kg, s.c.) suppressed AChE activity to less than 20% in all brain regions, compared to the controls, at 60 min following the exposure. Similarly, 60 min after a single acute dose of CF (1.5 mg/kg, s.c.), AChE was markedly depressed (% remaining activity: cortex, $10.02\% \pm 1.04\%$; amygdala, $18.18\% \pm 1.48\%$; and hippocampus, $12.75\% \pm 0.74\%$) (Gupta et al., 2007). At the time of high AChE inhibition and resultant severe seizure activity, significant increase in biomarkers of global free radical damage (F_2-IsoPs) and the selective peroxidation biomarker of neuronal membranes (F_4-NeuroPs) were seen in the brains of DFP- and CF-exposed animals. While twofold elevations are

seen in F_2-IsoPs levels, F_4-NeuroPs levels are more than fivefold higher than those of controls (Fig. 47.3). The results confirm the presence of oxidative damage in the cerebrum as a novel aspect of anti-AChE toxicity. The selective increase in F_4-NeuroPs indicates that neurons are specifically targeted by this mechanism.

DFP exposure also caused marked elevation in brain citrulline levels, which indicates NO/NOS activity (Gupta et al., 2001b, 2007). Control levels of citrulline are similar in the hippocampus (247.90 ± 4.10 nmol/g) and the amygdala (293.20 ± 6.90 nmol/g) (Gupta et al., 2007). Within 5 min of CF injection, the citrulline levels were elevated more than twofold in the investigated brain areas. Within 15 min of CF treatment, the levels of citrulline were significantly higher in both brain regions and were maximally elevated at 30 min postinjection (three- to fourfold). They remained elevated up to 60 min, but returned to control levels when measured 24 h later (Fig. 47.4). A similar response was seen following DFP exposure.

Many reports provide evidence that NO impairs mitochondrial/cellular respiration and other functions by inhibiting the activities of several key enzymes, particularly COx, thereby causing ATP depletion (Yang and Dettbarn, 1998; Dettbarn et al., 2001; Gupta et al., 2001a,b; Milatovic et al., 2001). Results from our experiments also showed that 1 h after DFP (1.5 mg/kg, s.c.) or CF (1.5 mg/kg, s.c.) treatment, the levels of ATP and PCr were significantly reduced in the hippocampus and amygdala (Fig. 47.5). With either DFP or CF treatment, the reduction in ATP and PCr levels was similar in the amygdala and hippocampus. During the course of these excitatory processes, a high rate of ATP consumption, coupled with the inhibition of oxidative phosphorylation, compromises the cell's ability to maintain its energy levels, and excessive amounts of ROS and RNS may be generated. Thus, the combination of impaired synthesis of ATP with its greater utilization during brain hyperactivity appears to result in a significant depletion of ATP. Three days after anti-AChE treatment, significant recovery of ATP and PCr is observed in discrete brain regions (Fig. 47.5). The rapid decrease in energy metabolites at the onset of seizures indicates early onset of mitochondrial dysfunction, in turn further increasing ROS production and neuronal injury.

An important question that emerged from previous studies is whether brain hyperactivity, such as seizures, first generates increases in ROS and then causes a decrease in high-energy phosphates (HEPs). The findings revealed that within 5−15 min after CF injection (the time required for onset and development of clinical signs), NO levels increased more than fivefold to sixfold in the cortex and more than twofold to threefold in the amygdala and hippocampus. The maximum increase in NO occurred at 30 min postinjection in all three brain regions. The data also revealed that the maximum decline in HEPs occurred 1 h after CF injection. This agrees with our previous data showing that a rapid and significant increase in NO precedes increases in lipid peroxidation, mitochondrial dysfunction, and loss of energy metabolites, as well as a reduction in COx activity and an increase in xanthine oxidase (Dettbarn et al., 2006). Therefore, the findings suggest that in the case of CF, the increase in ROS preceded the decrease in HEPs.

Seizures, convulsions, and CNS lesions are typical results of systemic application of sublethal doses of anti-

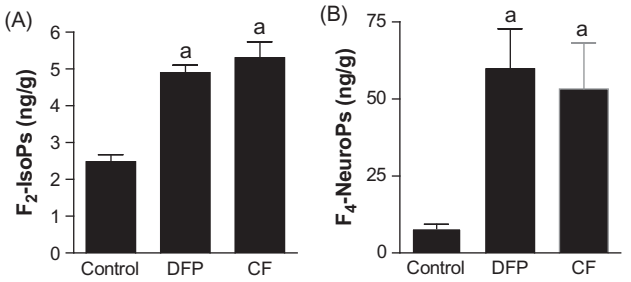


FIGURE 47.3 Effect of DFP (1.5 mg/kg, s.c.) and CF (1.5 mg/kg, s.c.) on F_2-IsoPs (A) and F_4-NeuroPs (B) levels in rat brain. Values are mean ± SEM ($n = 4-6$). [a]Significant difference between controls and DFP- or CF-treated rats ($P < .05$).

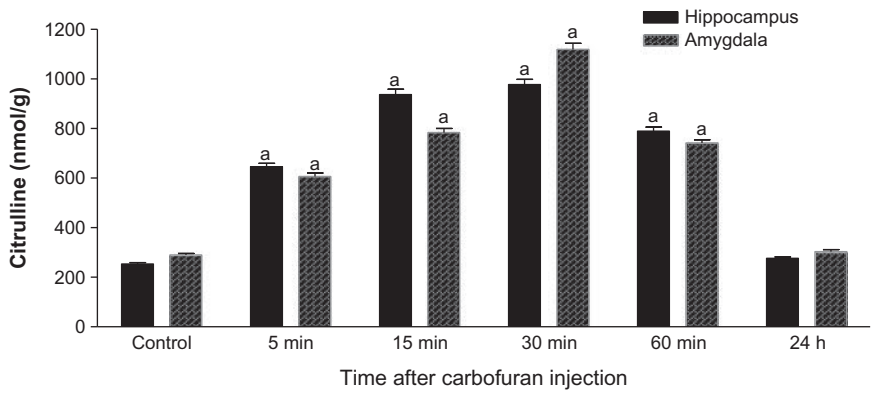


FIGURE 47.4 Citrulline levels in brain regions of rats intoxicated with an acute dose of CF (1.5 mg/kg, s.c.). Values of citrulline are presented as mean ± SEM ($n = 4-6$). [a]Significant difference between values from controls and CF-treated rats ($P < .05$).

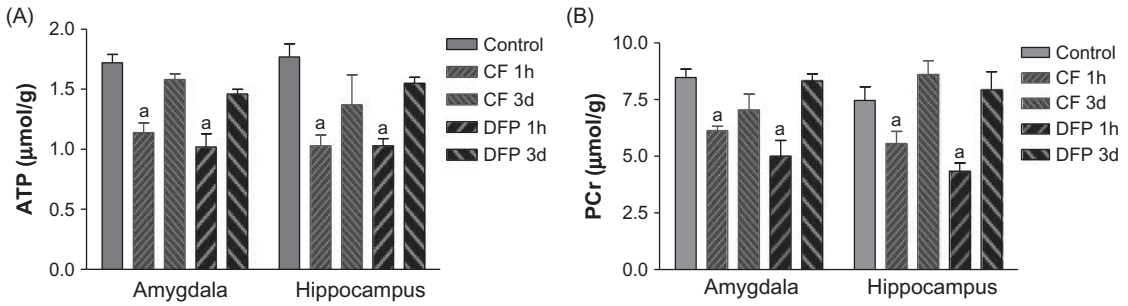


FIGURE 47.5 Levels of HEPs, ATP (A) and PCr (B), in the amygdala and hippocampus of rats intoxicated with an acute dose of CF (1.25 mg/kg, s.c.) or DFP (1.25 mg/kg, s.c.). Rats were sacrificed 1 h or 3 days after CF or DFP injection. Values of ATP and PCr are presented as mean ± SEM ($n = 4-6$). [a]Significant difference between values from controls and DFP- or CF-treated rats ($P < .05$).

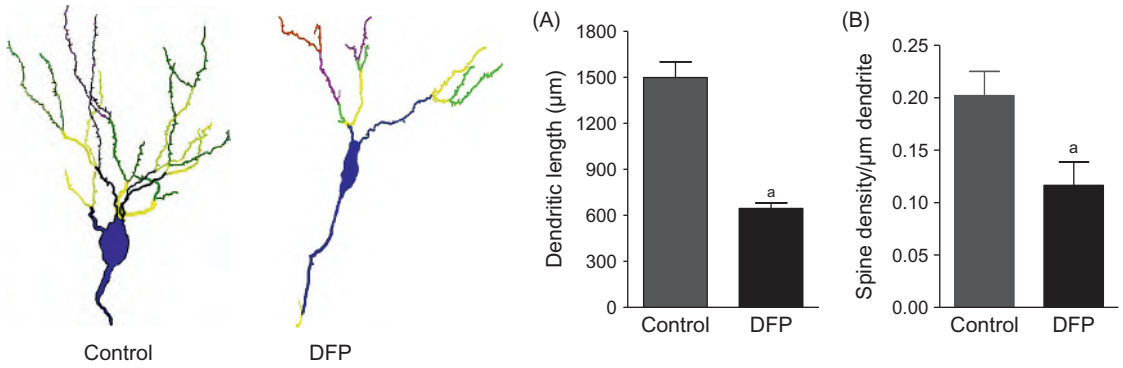


FIGURE 47.6 Morphology and quantitative determination of dendritic length (A) and spine density (B) of hippocampal pyramidal neurons from the CA1 sector of rats treated with saline (control) or DFP (1.5 mg/kg, s.c.) and sacrificed 1 h after the treatment. A total of 4–6 Golgi-impregnated dorsal hippocampal CA1 neurons were selected and spines counted by using the Neurolucida system. [a]Significant difference between controls and DFP-treated rats ($P < .05$). Treatment with DFP induced degeneration of the hippocampal dendritic system and decrease in the total length of the dendrite and spine density of hippocampal pyramidal neurons. Tracing and counting are done using the Neurolucida system at 100 × magnification under oil immersion (MicroBrightField, VT).

AChE agents (Sparenborg et al., 1992). The most consistent pathological findings in acute experiments include degeneration and cell death in the pyriform cortex, amygdala, hippocampus (where the CA1 region is preferentially destroyed), dorsal thalamus, and cerebral cortex. The early morphological changes in AChEI-induced SE include dendritic swelling of pyramidal neurons in the CA1 region of the hippocampus (Carpentier et al., 1991). Therefore, we have investigated whether seizure-induced cerebral oxidative damage in adult rats is accompanied by alterations in the integrity of the hippocampal CA1 dendritic system. Our results showed that anti-AChE induced early increases in biomarkers of global free radical damage (F_2-IsoPs) and the selective peroxidation biomarker of neuronal membranes (F_4-NeuroPs) was accompanied by dendritic degeneration of pyramidal neurons in the CA1 hippocampal area (Zaja-Milatovic et al., 2009). Anti-AChE-induced brain hyperactivity targeted the dendritic system with profound degeneration of spines and regression of dendrites, as evaluated by Golgi impregnation and Neurolucida-assisted morphometry (Fig. 47.6).

Rats injected with DFP show a significant decrease in total dendritic length and spine density compared to pyramidal neurons from the hippocampal CA1 area of control rats (Fig. 47.6). Taken together with the biochemical data presented previously, our results suggest that oxidative damage that selectively targets cerebral neurons is a hitherto-unrecognized aspect of anti-AChE toxicity. Results also revealed that anti-AChE exposure is associated with oxidative and nitrosative stress, alteration in energy metabolism, and consequent degeneration of pyramidal neurons from the CA1 hippocampal region of rat brain. Ultimately, the additive or synergistic mechanisms of cellular disruption caused by anti-AChE agents lead to cellular dysfunction and neurodegeneration.

47.5 Oxidative damage and dendritic degeneration following KA-induced excitotoxicity

Since excessive presynaptic release of glutamate and activation of NMDA and non-NMDA receptors have a

significant role in anti-AChE-induced neurotoxicity, we have investigated the role of glutamatergic excitation, oxidative injury, and neurodegeneration in the model of KA excitotoxicity. We have used intracerebroventricular (ICV) injection of KA, which is known as an experimental model for the investigation of cerebral vulnerability, particularly during acute brain disorders and SE (Schwob et al., 1980; Ben-Ari and Cossart, 2000). The study was designed to investigate whether F_2-IsoPs and F_4-NeuroPs formation correlated with the vulnerability of pyramidal neurons in the CA1 hippocampal area following KA-induced excitotoxicity. Our results showed that ICV KA-induced early increase in biomarkers of oxidative damage, F_2-IsoPs, and F_4-NeuroPs were accompanied by dendritic degeneration of pyramidal neurons in the CA1 hippocampal area.

Time-course changes in biomarkers of oxidative damage in the rat model of anti-AChE-induced seizures showed that the highest increase in F_2-IsoPs was evaluated 1 h after the injection of anti-AChE agent or 40 min after the beginning of seizure symptoms (Gupta et al., 2007). In the model of KA-induced excitotoxicity the earliest time point evaluated was 30 min since seizures start immediately after the ICV KA injection. Elevated levels of these in vivo markers of oxidative damage are in agreement with our previous findings (Montine et al., 2002c; Milatovic et al., 2005b; Gupta et al., 2007), as well as those of others (Patel et al., 2001), and indicate that KA injection leads to profound cerebral and neuronal oxidative damage in mice.

Our results also showed that the transient rise in F_2-IsoPs and F_4-NeuroPs is accompanied by rapid evolution of dendritic abnormalities, which becomes apparent due to a significant decrease in dendritic length and spine density of pyramidal neurons as early as 30 min post-KA injection. However, the recovery in both oxidative damage biomarkers at 60 min after the injection was not paralleled by the rescue of damaged neurons from the CA1 hippocampal area. Extended seizure activity (60 min) induced the same level of dendritic length and spine density decrease when compared to 30 min following KA injection (Table 47.1). Together, these data suggest that both oxidative stress and neurodegeneration occur as an early response to seizures, but they do not determine whether oxidative stress is a cause or an effect of seizure-induced CA1 cell damage. Neuronal damage processes triggered by sustained seizure activity may occur as a continuum, last longer than formation of oxidative lipids, and, although not evident by the markers, may already be in progress when the peak increases in F_2-IsoPs and F_4-NeuroPs occur. Thus, we investigated dynamic changes in lipid peroxidation and dendritic structures immediately after seizures occur, but future studies over the longer period should be able to determine the long-term course of these spine and dendritic changes. It is very likely that the spine loss seen in our study is the initial phase of more chronic spine loss and progressive neurodegeneration reported in other studies (Muller et al., 1993; Jiang et al., 1998; Zeng et al., 2007).

In vivo data have also established that KA induced a significant increase (more than twofold) in citrulline concentrations 30 min following the injection (Zaja-Milatovic et al., 2008). Although we did not determine whether increased citrulline originated from a combination of NOS isozymes or one in particular, our data agree with the results from the models of anti-AChE toxicity and activated innate immunity (Milatovic et al., 2003, 2004; Gupta et al., 2007) and indicate that a subset of NOS activity also contributes to cerebral oxidative damage in the model of KA-induced excitotoxicity.

47.6 Suppression of seizure-induced oxidative injury and neurodegeneration

47.6.1 Antioxidants

Antioxidants [e.g., vitamins, glutathione (GSH), selenium, zinc, creatine, and arginine] and antioxidant enzymes (e.g., superoxide dismutase, catalase, GSH reductase, and GSH peroxidase) exert synergistic actions in scavenging free radicals. A large body of literature (e.g., Fang et al., 2002) supports the notion that antioxidants play an

TABLE 47.1 Cerebral concentrations of F_2-IsoPs and F_4-NeuroPs and dendritic degeneration of hippocampal pyramidal neurons following KA-induced seizures in mice.

	F_2-IsoPs (ng/g)	F_4-NeuroPs (ng/g)	Dendritic length (μm)	Spine density (number/100 μm dendrite)
Control	3.07 ± 0.05	13.89 ± 0.58	1032.10 ± 61.41	16.45 ± 0.55
KA 30 min	4.81 ± 0.19[a]	34.27 ± 2.71[a]	363.44 ± 20.78[a]	8.81 ± 0.55[a]
KA 60 min	3.40 ± 0.18	18.55 ± 1.26	425.71 ± 23.04[a]	7.44 ± 0.56[a]

Data from KA-exposed mice were collected 30 or 60 min postinjection.
[a]*One-way ANOVA showed P < .0001 for each end-point. Bonferroni's multiple comparison test showed significant difference (P < .001) compared to vehicle-injected control.*

important role in preventing many human diseases (e.g., cancer, atherosclerosis, stroke, rheumatoid arthritis, and neurodegeneration). Vitamin E has been recognized as one of the most important antioxidants. Vitamin E inhibits ROS-inducing generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFAs in membrane phospholipids, from oxidative damage of cellular proteins and DNA, and from membrane degeneration (Topinka et al., 1989; Azlina et al., 2018; Simioni et al., 2018). Vitamin E mainly acts as a chain-breaking antioxidant and radical scavenger, protecting cell membranes against oxidative damage (Van Acker et al., 1993). In addition, vitamin E regulates ROS production (Chow et al., 1999), maintains oxidative phosphorylation in mitochondria, and accelerates restitution of high-energy metabolites (Punz et al., 1998). Decreased levels of vitamin E in response to hyperoxia or treatment with convulsants reported in studies (Onodera et al., 2003; Mori et al., 2004; Rauca et al., 2004) suggest that vitamin E in the brain is consumed to prevent oxidative damage. Vitamin E also prevented metasystox (OP insecticide)-induced changes in lipase activity and lipid peroxidation in the brain and spinal cord of rats (Tayyaba and Hasan, 1985).

A synthetic spin-trapping agent such as phenyl-*N*-*tert*-butylnitrone (PBN) is also capable of scavenging many types of free radicals. This compound is widely used to trap ROS in a variety of physical, chemical, and biological studies using electron paramagnetic resonance spectrometry. PBN is known to be concentrated in the mitochondria, where it reacts with ROS and forms stable adducts, and thereby maintains normal levels of energy metabolites. Numerous in vitro and in vivo experiments have shown the beneficial effects of PBN on the prevention of neuronal degeneration. Protective effects are described in experimental models of brain ischemia/reperfusion (Carney and Floyd, 1991; Fetcher et al., 1997; Gido et al., 1997), excitotoxicity (Lancelot et al., 1997; Milatovic et al., 2002), inhibition of NOS induction (Miyajima and Kotake, 1995), and in different models of seizures (He et al., 1997; Thomas et al., 1997). Additional findings also corroborate that PBN effectively prevents neurodegeneration in Parkinson's disease (Sack et al., 1996; Frederiksson et al., 1997), Alzheimer's disease, and anti-AChE neurotoxicity (Sack et al., 1996; Gupta et al., 2001a,b). Thus, PBN has been proven to rescue neurons in multiple experimental injury models. Other pharmacological properties of spin-trapping agents have been described that could influence the outcome of oxidant injury. These have been described for PBN as reversible Ca^{2+} channel blockade in vascular muscle, causing vasodilatation (Anderson et al., 1993); direct effect on striatal function, including inhibition of excitation−contraction coupling; and induction of hypothermia (Pazos et al., 1999).

Previous studies have shown that antioxidant pretreatment suppressed DFP- or CF-induced alterations in HEP, their metabolites, and citrulline levels, supporting the possibility that increased generation of ROS/RNS contributes to the depletion of energy phosphates (Gupta et al., 2001a,b). PBN or vitamin E treatment alone did not alter the levels of HEPs, their major metabolites, or citrulline in any of the brain regions. Vitamin E pretreatment suppressed the depletion of HEP and their metabolites and increased citrulline levels without preventing seizures (Gupta et al., 2001a). The protective efficacy provided by vitamin E against DFP- or CF-induced changes in energy metabolites was of varying degrees in different brain regions and could partly be due to pharmacokinetic variables involved in attaining different levels of vitamin E in different brain regions. However, PBN pretreatment 1 h before the anti-AChE agent protected mitochondria and maintained the cellular level of high-energy metabolites, but it also prevented DFP- or CF-induced convulsions and seizures (Gupta et al., 2001a,b). This could primarily be due to a protective interaction of PBN with AChE, sufficient to protect a critical fraction of AChE against phosphorylation by DFP or carbamylation by CF (Zivin et al., 1999; Milatovic et al., 2000a,b). We have also shown that AChE inhibitor-induced increases in NO (citrulline) were significantly prevented by PBN and by vitamin E (Gupta et al., 2001a). There is evidence that suggests that PBN inhibits the induction of inducible NOS (iNOS) by reducing the expression of iNOS protein (decrease in mRNA expression), thus preventing the overproduction of NO (Miyajima and Kotake, 1997).

The efficacy of the spin-trapping agent PBN and the antioxidant vitamin E was tested to suppress the increase in NO and lipid peroxidation and prevent neurodegeneration of pyramidal neurons in the CA1 hippocampal area in the model of KA-induced excitotoxicity (Zaja-Milatovic et al., 2008). Vitamin E or PBN alone did not alter basal citrulline and F_4-NeuroPs levels or dendritic arborization. However, vitamin E and PBN suppressed KA-induced increases in citrulline and cerebral and neuronal markers of oxidative damage, F_2-IsoPs and F_4-NeuroPs, respectively (Figs. 47.7 and 47.8).

Importantly, vitamin E and PBN completely suppressed both reduction in dendrite length and reduction in spine density of pyramidal neurons from the CA hippocampal area from KA-exposed mice (Fig. 47.9).

A close concordance was found between these results, showing that protection of the cerebrum from neuronal oxidative damage also protected hippocampal CA1 pyramidal neurons from dendritic degeneration. These agents did not alter kainate-induced seizure severity, indicating that the protective effect of vitamin E and PBN is most likely mediated by scavenging ROS and preventing lipid peroxidation and consequent neuronal damage, not by a

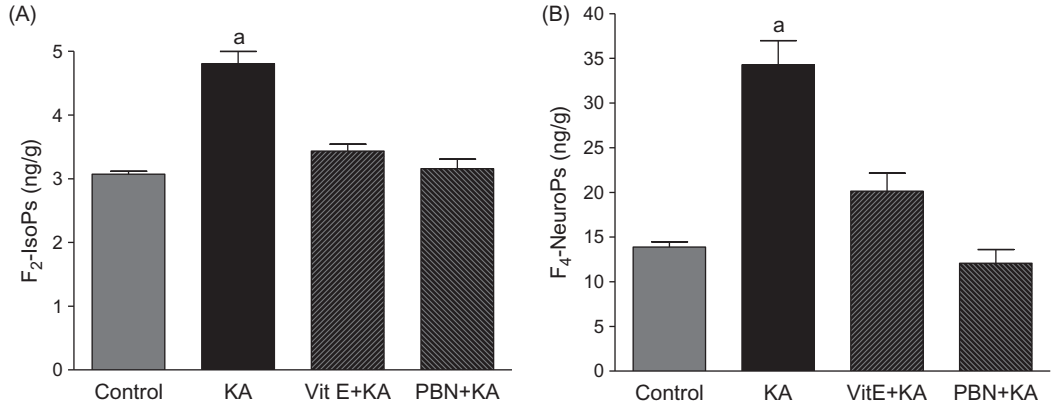


FIGURE 47.7 Ipsilateral cerebral F_2-IsoPs (A) and F_4-NeuroPs (B) concentrations following ICV KA with or without vitamin E (Vit E) or PBN pretreatment. Brains from mice exposed to KA were collected 30 min postinjection ($n \geq 5$ for each group). One-way ANOVA had $P < .0001$ with Bonferroni's multiple comparison tests significant for KA versus control, vitamin E + KA or PBN + KA treatment.

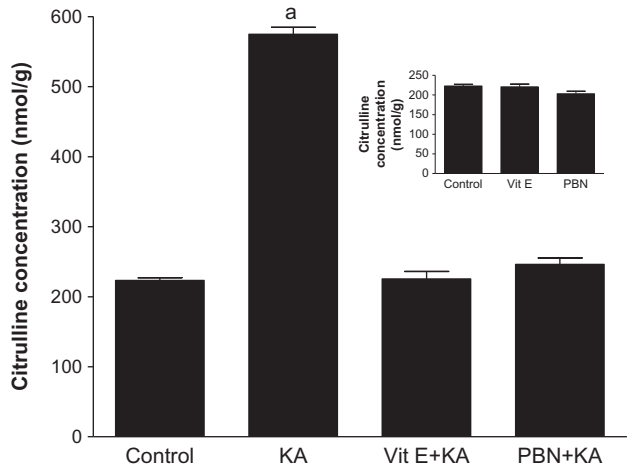


FIGURE 47.8 Ipsilateral cerebral citrulline concentrations following ICV KA with or without vitamin E or PBN pretreatment. Brains from mice exposed to KA were collected 30 min postinjection ($n \geq 5$ for each group). One-way ANOVA had $P < .001$ with Bonferroni's multiple comparison tests significant for KA versus control, vitamin E + KA, or PBN + KA treatment.

specific effect on seizures per se. Furthermore, since antioxidants minimize lipid peroxidation following an increase in α-tocopherol and PBN, then a parallel reduction in neuronal damage provides strong evidence that oxidative stress and lipid peroxidation in a causal way mediate seizure and the corresponding injury. One limitation to the potential therapeutic application of this type of substance is that, to be effective, the drugs need to be administered prophylactically before the onset of seizures. Future research should address the efficacy of these agents in preventing seizure-induced oxidative and dendritic changes and potentially reducing resultant neurocognitive deficits when they are administered at higher concentrations, either during or possibly even after seizures.

47.6.2 *N*-methyl-D-aspartate receptor antagonist (memantine)

As excitotoxicity-induced neuronal damage in the model of anti-AChE-induced seizures is explained by the excessive release of glutamate that activates both NMDA and non-NMDA postsynaptic receptors, antagonism of the excitotoxicity mechanism may protect the CNS from the deleterious effects of anti-AChE agents. Several NMDA receptor antagonists have been shown to exert anticonvulsant effects against nerve agent-induced seizures when administered either as a pretreatment or after the seizure has been initiated, usually terminating the convulsions after an initial period of epileptical activity (Shih, 1990; Sparenborg et al., 1992). NMDA receptor antagonists do not modify the events responsible for the early phase of the seizure, but they block the subsequent recruitment of glutamate receptor activation, and hence the maintenance of seizure activity and irreversible functional and structural brain damage.

Among promising candidates as antidotes against CNS intoxication by OP nerve agents, memantine has been shown to pose both antiexcitotoxic and antiepileptic properties. Memantine is an uncompetitive NMDA receptor antagonist, clinically used for the treatment of Alzheimer's disease, Parkinson's disease, and spasticity, in the absence of serious side effects (Lipton, 2005; Ozsuer et al., 2005; Wang and Redd, 2017; Folch et al., 2018). From a series of rat in vivo experiments, it is evident that preadministration of memantine significantly protects AChE activity from inhibition caused by AChE inhibitors, including OP and CM insecticides and OP nerve agents (Gupta and Kadel, 1990; Gupta and Dettbarn, 1992; McLean et al., 1992; Gupta and Dekundy, 2005). By now, it has been well established that memantine exerts various pharmacological effects by multiple pharmacological mechanisms: (1) blockage of

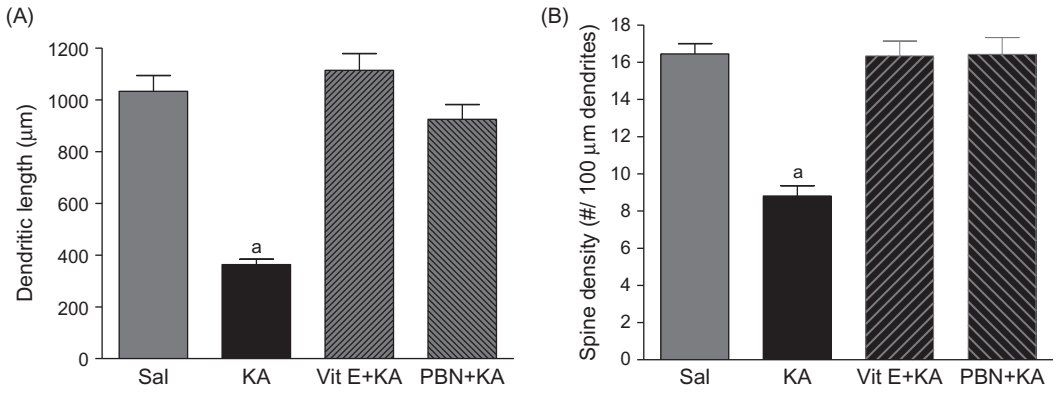


FIGURE 47.9 Dendritic length (A) and spine density (B) of pyramidal neurons from the CA1 hippocampal area of mice following ICV KA with or without vitamin E or PBN pretreatment. Brains from mice exposed to KA were collected 30 min postinjection ($n \geq 5$ for each group). One-way ANOVA had $P < .001$ with Bonferroni's multiple comparison tests significant for KA versus control, vitamin E + KA or PBN + KA treatment.

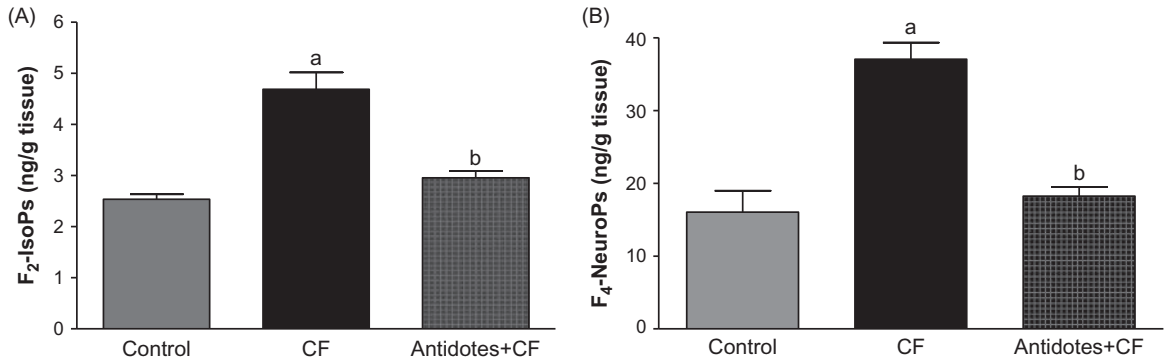


FIGURE 47.10 Cerebral F_2-IsoPs (A) and F_4-NeuroPs (B) concentrations following CF (1.5 mg/kg, s.c.) with or without antidote pretreatment (memantine, 18 mg/kg, and atropine, 16 mg/kg, given prophylactically, 60 and 15 min, respectively, before CF administration). Brains from rats exposed to CF were collected 60 min postinjection ($n \geq 5$ for each group). One-way ANOVA had $P < .0001$ with Bonferroni's multiple comparison tests significant for CF versus control (a), and for CF versus antidote + CF (b).

nicotinic ACh receptor-ion channel complex (Masuo et al., 1986), (2) reduced reflex excitability of both flexors and extensors (Wand et al., 1977), (3) prevention of neural hyperexcitability (McLean et al., 1992), (4) central muscle relaxation (Grossman and Jurna, 1997), and (5) prevention of AChE inhibitor-mediated energy loss from muscle cells (Milatovic et al., 2005a). Memantine is also able to prevent the pathogenic calcium influx caused by continuous mild activation by low-level glutamate. On the other hand, memantine allows the physiological activation of the NMDA channels by high concentrations of glutamate, a phenomenon necessary for synaptic plasticity underlying normal learning and memory (Parsons et al., 1999).

Previous studies have also demonstrated that memantine treatment significantly reduces lipid peroxidation (Fig. 47.10) and alterations in citrulline and HEP levels in muscles and brain of rats intoxicated with CF (Milatovic et al., 2005a; Gupta et al., 2007; Zaja-Milatovic et al., 2009).

No significant alterations in biomarkers of neuronal damage, citrulline, HEP, and their metabolite levels were seen in any of the brain regions receiving memantine and atropine. In addition, memantine and atropine exposure did not induce any alteration in neuronal morphometry, but when given as pretreatment, it did provide protection against CF-induced morphometric changes in hippocampal neurons (Fig. 47.11). Memantine, in combination with atropine, completely suppressed the reduction in both dendrite length and in spine density of pyramidal neurons from the CA1 hippocampal area from CF-exposed rats (Fig. 47.11).

In conclusion, the data demonstrated that synergistic mechanisms of cellular disruption caused by anti-AChE agents led to cellular dysfunction and neurodegeneration. It has also been demonstrated that preventing CF-induced neuronal hyperactivity by pretreatment with memantine and atropine blocks pathways associated with oxidative damage in rat brain. The documented ability of memantine therapy to reduce free radical generation and lipid

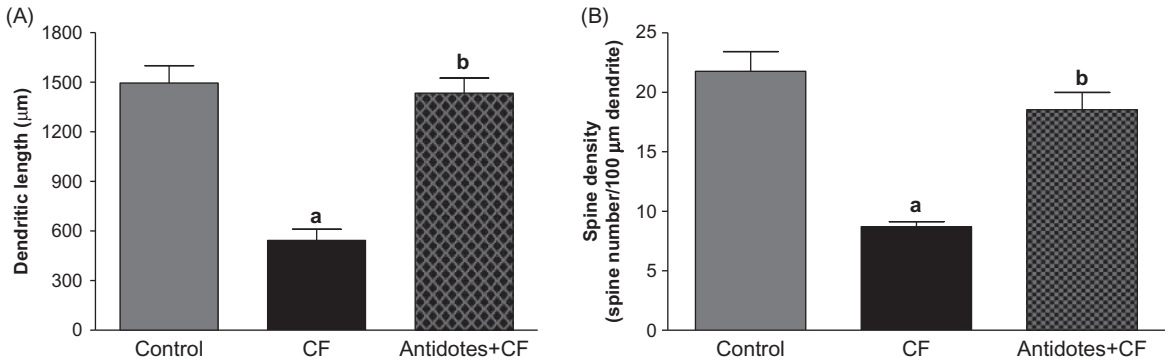


FIGURE 47.11 Dendritic length (A) and spine density (B) of pyramidal neurons from the CA1 hippocampal area of mice following CF (1.5 mg/kg, s.c.) with or without antidote pretreatment (memantine, 18 mg/kg and atropine, 16 mg/kg given prophylactically, 60 and 15 min, respectively, before CF administration). Brains from rats exposed to CF were collected 60 min postinjection ($n \geq 5$ for each group). One-way ANOVA had $P < .0001$ with Bonferroni's multiple comparison tests significant for CF versus control (a), and for CF versus antidote + CF (b).

peroxidation, prevent HEPs, and attenuate the morphological injury provides further support for the role of ROS and RNS in anti-AChE-induced seizures.

47.7 Concluding remarks and future directions

Exposure to OP nerve agents induces seizures, rapidly progressing to SE and profound structural brain damage. The progression of events includes initial high cholinergic activity followed by activation of glutamatergic neurons as a result of release of glutamate. Moreover, glutamate stimulates the continuous release of ACh, contributing to further excitatory stimulation, prolongation of the seizures, and excitotoxic neurodegeneration in vulnerable brain areas. The ensuing neuronal damage is thought to result from intense transient influx of calcium, which leads to mitochondrial functional impairment, cytochrome c-inactivation, depletion of ATP, simultaneous formation of free radical species, and oxidative stress. Therefore, control of excitotoxicity and oxidative stress, better understanding of the mechanisms of noncholinergic-mediated activities, and pathways that protect or promote neuronal survival are essential for the development of efficacious treatments and preventive therapies associated with OP exposures.

We have explored mechanisms associated with OP-induced neurotoxicity by probing their effects on oxidative stress and associated dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. We have also investigated different pathways to attenuate biomarkers of oxidative damage associated with anti-AChE exposure and the extent to which such attenuation is accompanied by rescue from neurodegeneration. Results from our studies suggest that vitamin E, PBN, and memantine efficiently suppress oxidative injury. Future studies should be directed at deciphering the mechanisms of protection, addressing the ability of these agents to attenuate OP neurotoxicity via radical scavenging, AChE inhibition, suppression of neuroinflammation, glutamate antagonism, or any combination of these. Additional studies should also determine whether a combination of these treatments improves the therapeutic index against OP poisoning (compared to administration of each alone). Complementary studies should also investigate not only the prophylactic, but also the therapeutic effects of these neuroprotectants. Successful identification of safe and effective neuroprotectants that suppress noncholinergic activities associated with anti-AChE exposure will provide new pharmacological modalities to protect and treat both the acute and delayed effects of nerve agent poisoning.

Acknowledgment

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Chapter 48

Blood–brain barrier damage and dysfunction by chemical toxicity

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48.1 Introduction

Direct evidence for the existence of the blood–brain barrier (BBB) came at the dawn of the 20th century from the observations of Nobel Laureate Paul Ehrlich. When he injected colored dyes, including trypan blue, into the bloodstream, they leaked out of capillaries in most regions of the body to stain the surrounding tissue, but the brain and spinal cord remained unstained. Further support for the existence of the BBB came from the work of the Russian neurophysiologist Lina Stern in 1921, who called this barrier a "hematoencephalic" barrier. Since then, doubts have been cast over the actual existence of the BBB, until the emergence of electron microscopic data demonstrating the presence of the tight junctions between the endothelial cells that form brain capillaries (Abbott et al., 2010; Palmer, 2010). The BBB is formed and maintained through a dynamic interaction between cerebral and endothelial cells constituting the anatomical basis of the BBB and other neighboring cells, such as astroglia, pericytes, perivascular microglia, and neurons (Deli et al., 2005). The cross-talk between these cells endows endothelial cells with a unique BBB phenotype comprising not only the morphological barrier of endothelial tight junctions, but also the enzymatic and metabolic barriers, as well as the uptake and efflux transport systems (Abbott et al., 2010).

The term BBB describes a series of mechanisms that control the internal environment of the brain (Saunders et al., 2008). Stability of this environment is essential for normal brain development and function. Underlying the cellular mechanisms that determine the brain's internal environment is a fundamental physical barrier at the level of intercellular tight junctions between cells forming the interface between blood and brain (BBB) and in the choroid plexuses (blood–cerebrospinal fluid barrier, BCSFB). These tight junctions severely attenuate or occlude movement of chemicals and proteins through intercellular spaces between endothelial cells in the BBB and epithelial cells in the choroid plexus BCSFB.

The BBB formed by the endothelium of cerebral blood vessels is one of the three main barrier sites protecting the central nervous system (CNS). The barrier is not a rigid structure, but rather a dynamic interface with a range of interrelated functions, resulting from extremely effective tight junctions, transendothelial transport systems, enzymes, and regulations of leukocyte permeation, which thereby generates the physical, transport, enzymatic, and immune regulatory functions of the BBB (Abbott and Friedman, 2012). In addition, recent studies have revealed important stages, cell types, and signaling pathways involved in BBB development. Several enzymes (monoamine oxidase, epoxy hydrolase, endopeptidases, acetylcholinesterase, DOPA decarboxylase, γ-glutamyl transpeptidase) are known to be present in endothelial cells and are important elements of the BBB phenotype constituting the so-called metabolic barrier, and they participate in the regulation of brain penetration of drugs (Fenstermacher and Neuwelt, 1989; Pardridge, 2002). The role of the BBB as a metabolic barrier was further ascertained by the presence of mitochondria in cerebral endothelial cells (Fenstermacher and Neuwelt, 1989). Thus, the BBB may be considered a physical as well as a metabolic barrier.

Because the involvement of brain barriers is apparent in neurodegenerative diseases, neurotrauma, chemical-induced neurotoxicity, and delivery of drugs of use and abuse, renewed interest in this area of research has emerged (Neuwelt et al., 2008; Palmer, 2010).

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In physiological conditions, the BBB regulates the exchange of nutrients, waste, and immune cells between the blood and the nervous tissue of the CNS and is the most important component preserving CNS homeostasis and neuronal function (Abbott et al., 2010). Dysfunctional brain barrier mechanisms contribute to the pathology of neurological conditions, ranging from trauma to neurodegenerative diseases, and provide obstacles for successful delivery of potentially beneficial pharmaceutical agents (Saunders et al., 2008; Palmer, 2010; Erickson and Banks, 2013). Interestingly, modulations in the BBB can be the cause and/or consequence of non-CNS diseases, such as diabetes, chronic inflammatory pain, and obesity, but they are not discussed in this chapter because of chapter size constraints. This chapter describes, in brief, the structure and function of the BBB and modulations of its components and permeability by chemical warfare agents (CWAs) and other toxicants, Gulf War illness (GWI), stress, blasts, excitotoxicity, and neurodegenerative diseases.

48.2 Structure and function of the BBB

The brain develops and functions within a strictly controlled environment resulting from the coordinated action of different cellular interfaces located between the blood and the extracellular fluids (interstitial fluid and the cerebrospinal fluid) of the brain (Strazielle and Ghersi-Egea, 2013). The barrier between the blood and the brain or spinal cord parenchyma proper, referred to as the BBB, is formed by the endothelium of the cerebral microvessels. Several layers exist between the blood and brain: capillary endothelial cells, a basement membrane consisting of type IV collagen, fibronectin and laminin that completely cover the capillaries, pericytes embedded in the basement membrane, and glia/astrocytes that surround the basement membrane (Fig. 48.1). Each of these layers could potentially restrict the movement of solutes (Hawkins et al., 2006; Alvarez et al., 2013).

de Boer et al. (1998) described that the BBB has narrow tight junctions, has no intercellular clefts, has minor pinocytotic activity, is not fenestrated, has a continuous basement membrane, contains many mitochondria, and has high electrical resistance (1500−2000 $\Omega \times cm^2$). Next to the microvascular endothelial cells, pericytes, microglia, and neurons influence the functionality of the BBB, and there are also leukocytes (lymphocytes and monocytes) and, in the surrounding larger vessels, perivascular macrophages and mast cells. In addition to endothelial and epithelial cells, astrocytes and pericytes are involved in structure, functions, and regulation of brain barriers (Abbott et al., 2010; Armulik et al., 2010; Daneman et al., 2010; Wilhelm et al., 2011; Mizee et al., 2013).

The BBB is to be distinguished from a second barrier component located between the blood and the ventricular CSF, and thus is called the blood−CSF barrier (BCSFB). The BCSFB interface is formed by the tight epithelium of the choroid plexuses, which are specialized structures projecting in all four ventricles of the brain and are responsible for the active secretion of CSF. The BBB develops a large surface area of exchange between the blood and the neutrophil, with an average of 100 cm^2/g of brain tissue in the adult mammal. In a 1-month-old rat, the apical surface area in contact with CSF has been estimated to be 75 cm^2 and close to the surface area developed by the BBB (Keep and Jones, 1990).

BBB tightness is maintained by various intercellular junctions, such as tight junctions, adherence junctions, gap junctions, and syndesmos, from which the tight junctions seem to be the most important ones in restricting passive hydrophilic transport. The BBB presents more possibilities for the transport of small molecules (molecular weight 400−600 Da) compared with large ones, keeps the composition of electrolytes constant in the interstitial fluid of the brain, and prevents the passage of water-soluble drugs and proteins from the blood to the brain.

The BBB closely regulates the exchange of molecules in and out of the brain parenchyma to maintain its optimal homeostasis for a chemical environment that allows adequate brain functioning (de Vries et al., 2012). The ionic stability, neurochemical environment, and normal functioning of the brain are crucially dependent on the integrity of the brain barrier systems (Saunders et al., 2008). Without this stability, complex functions performed by the brain would be impossible. Saunders et al. (2008) further emphasized that dysfunction of brain barrier mechanisms in a variety of pathologies is more than a disruption of the normal (tight junction) diffusion restraint and that such dysfunction might be a part of the disease process, rather than a consequence. Erickson and Banks (2013) described that the BBB dysfunction is a cause as well as a consequence of neurological diseases, such as Alzheimer disease (AD).

48.3 In vivo and in vitro models to study the BBB

The movement of compounds from the circulating blood into the brain is strictly regulated by the brain capillary endothelial cells, which constitute the BBB. The importance of the BBB is not only in the passage of toxicants but also in therapeutic drugs and antidotes. Brain capillary endothelial cells are connected to each other by continuous tight junctions and have a low number of pinocytotic vesicles. Several proteins (such as P-glycoproteins) and enzymes (such as monoamine oxidase, DOPA

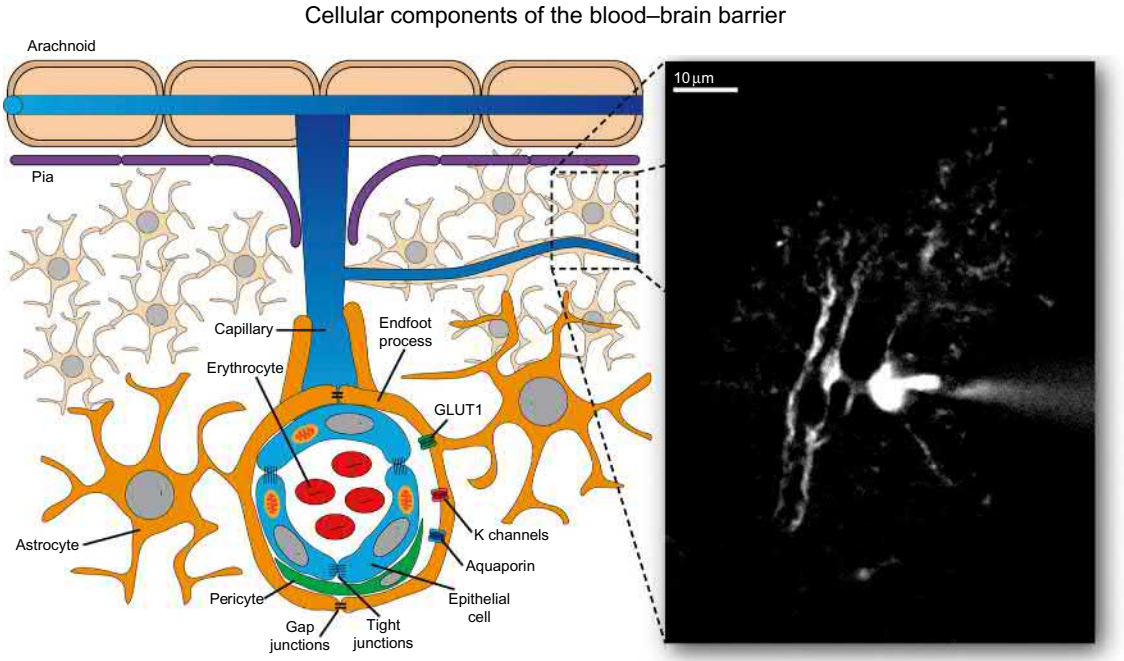

FIGURE 48.1 The BBB is a multicellular interface.
The brain is a highly vascularized organ; blood vessels in the arachnoid space enter the brain and form a network of highly branched capillaries. Vascular epithelial cells are linked together through tight and adherens junctions. These intercellular junctions restrict the entry of bloodborne molecules from entering the CNS and form the BBB. Interactions between the epithelial cells are maintained by astrocytes, a type of glial cell that surrounds more than 99% of the vascular epithelium. Astrocytes ensheath blood vessels with specialized processes called endfoot processes, which express a variety of transporter proteins to maintain osmotic balance and uptake glucose from the blood. This intimate relationship between astrocytes and the vasculature is illustrated in the inset figure, which shows an astrocyte in an acute brain slice filled with a fluorescent dye. The patch pipette and blood vessel are visible to the right and left of the astrocyte, respectively. The buffering capacity of astrocytes is dramatically improved through a series of gap junctions between neighboring astrocytes.

decarboxylase, acetylcholinesterase, and others) are expressed by brain endothelial cells and constitute the so-called metabolic barrier. Because of these characteristics, the BBB limits or impairs the delivery of certain drugs to the CNS. Therefore, to understand the crossing of compounds of various classes, drugs, and antidotes, various in vivo and in vitro models have been developed.

48.3.1 In vivo model

Birngruber et al. (2013) reported a cerebral open-flow microperfusion as a new membrane-free technique for measuring substance transport across the intact BBB in Sprague-Dawley rats. This in vivo technique is based on a probe that is inserted into the brain, thereby rupturing the BBB. The BBB is usually reestablished within 15 days, which then allows sampling of interstitial brain fluid under physiological conditions. This technique allows monitoring of BBB permeability, which can be useful for measuring pharmacokinetics across the BBB and pharmacodynamics in the brain. Using tracers, such as Evans blue (EB), horseradish peroxidase, and [^{131}I]albumin, breakdown of the BBB in humans and experimental animals has been studied under many conditions, such as hypoglycemia, hypertension, seizures/convulsions, and inflammation (Öztaş and Couraud, 1996).

48.3.2 In vitro models

In vitro reconstituted models of the BBB from different mammalian species have been used since the late 1970s. However, their comparison is difficult because of the different species and methods used for isolation, culture, coculture, and characterization of the models. Lundquist et al. (2002) confirmed that the epithelial cells might not represent a valid and reliable in vitro BBB model, because results obtained on epithelial monolayers correlated poorly with in vivo BBB permeability values. Bowman et al. (1983) introduced the first in vitro BBB filter model. The insert was made of nylon mesh and polycarbonate tubing, and bovine brain endothelial cells were seated on it for studying the effect of calcium-free medium and osmotic shock on sucrose flux. Since then, a variety of chambers and inserts from different materials and with diverse pore size have become commercially available. Garberg et al. (2005) used an in vitro model for

BBB permeability based on the use of a continuous cell line and to investigate the specificity of this model. These authors developed a coculture procedure that mimics the in vivo situation by culturing brain capillary endothelial cells on one side of a filter and astrocytes on the other. Under these conditions, endothelial cells retain all the endothelial cell markers and characteristics of the BBB, including tight junctions and enzyme activities (γ-glutamyl transpeptidase and monoamine oxidase). Raub (1996) identified the signaling pathways involved in the barrier-enhancing effects of C6 glioma cells, suggesting that the action is not mediated through cAMP, but rather by protein kinase C (PKC) activation via phospholipase D, independent of intracellular calcium increase.

Deli et al. (2005) presented permeability data from various in vitro BBB models by measuring transendothelial electrical resistance (TEER) and by calculation of permeability coefficients for paracellular or transendothelial tracers. These authors summarized the results of primary cultures of cerebral microvascular endothelial cells or immortalized cell lines from bovine, human, porcine, and rodent origins. This also described the effect of coculture with astroglia, neurons, mesenchymal cells, blood cells, and conditioned media, as well as the physiological influence of serum components, hormones, growth factors, lipids, and lipoproteins on the BBB function.

The strong correlation between in vivo (Oldenhorf method) and in vitro (coculture) drug transport, the relative ease with which such cocultures can be produced in large quantities, and the reproducibility of the system provide evidence for an efficient system for the screening of drugs that are active in the CNS (Dehouck et al., 1997). These authors suggested that the coculture method is a useful system for investigating passive diffusion, carrier-mediated transport, and P-glycoprotein (Pgp)-dependent drug transport. The in vitro permeabilities of propranolol and cyclosporine A were parallel with indications from in vivo extraction, showing that transporters and Pgp are expressed in the coculture system. For further details, readers are referred to the work by Deli et al. (2005), who reviewed various in vitro models covering bovine, human, porcine, and rodent brain endothelial cell-based systems. Bovine systems provide a high yield of brain endothelial cells sufficient for pharmacological screening, and they are widely used in basic as well as in applied research. Mouse brain yields the least endothelial cells compared with other species.

Some examples of the modulators of BBB permeability in in vitro models are: both cAMP elevator peptide hormone adrenomedulin and calcitonin gene-related peptide decrease paracellular permeability; a glucocorticoid hormone, hydrocortisone, improves the barrier properties; insulin exerts a tightening effect on tight junctions; and catecholamines (adrenaline and noradrenaline) increase the sodium fluorescein flux.

In essence, in vitro models have been widely used in pharmacological research for screening drugs and drug candidate molecules for either modifying BBB permeability or investigating brain penetration (Deli et al., 2005; Wilhelm et al., 2011). This area of research is very important for permeability screening during drug development in the pharmaceutical industry.

48.4 Gender differences in the BBB

For more than three decades, evidence has suggested that the BBB differs in males and females in terms of morphology, metabolism, and permeability. Interest arose from previous studies suggesting that women had higher cerebral blood flow than men, and the differences were more pronounced in the frontal regions (Mathew et al., 1986). Follow-up studies further confirmed the finding of higher cerebral blood flow in females as compared with males (Rodriguez et al., 1988).

In animal studies, although Saija et al. (1990) found no substantial difference in the permeability of the BBB between male and female rats, fluorescein penetrated to a greater extent in the brains of females as compared with males, but only into those regions that reside outside the BBB. Öztaş (1998) investigated the gender effects on BBB permeability during bicuculline-induced seizures in female and male rats and found the extravasation of EB-stained albumin in a more pronounced manner in the brains of females as compared with males. In a similar study, Öztaş and Couraud (1996) noted that disruption of BBB permeability during pentylenetetrazol-induced seizures was asymmetric between the right and left hemispheres in female rats but not in male rats. Interestingly, ovariectomy decreased BBB permeability during seizures, suggesting the involvement of estrogen because endothelial cells have been shown to contain estrogen receptors (Colburn and Buonassivi, 1978).

Furthermore, the distribution of serotonin is different in the brain and cerebral endothelial cells of males and females (Fischette et al., 1983), and serotonin is an important modulator of BBB permeability (Sharma and Dey, 1986). The sex hormone-related differences in the serotonin and other neurotransmitter levels in endothelial cells may cause different responses under pathological conditions. Öztaş (1998) hypothesized that if BBB permeability can increase more easily in females, then this increased breakdown may result in higher incidences of neurodegenerative diseases, such as AD and multiple sclerosis (MS) in women.

48.5 The BBB in young and adult brains

The structure and function of the BBB substantially differ in young and adult brains. In general, the BBB is immature in fetuses and newborns. This may be partly because of the fact that the blood vessels in the immature brain are more fragile than in the adult. Astrocytes in the developing brain are also responsible for the induction of tight junctions in the cerebral endothelial cells as well as other features of brain barrier mechanisms (Janzer and Raff, 1987; Saunders and Habgood, 1999). Saunders et al. (1999) provided evidence that barriers to proteins at blood–brain and blood–CSF interfaces (tight junctions) are present from very early in development.

Immunocytochemical and permeability findings revealed that proteins are largely excluded from extracellular space in the developing brain. In addition to tight junctions present at the BBB and BCSFB, the immature brain also has a mixture of other junctions present at the outer CSF–brain barrier (plate junctions, strap junctions, and wafer junctions). These barriers are not present in the adult (Saunders et al., 1999). It was suggested that both the functional and morphological properties of the BBB develop progressively from the onset of intraneural vascularization. However, the morphological characteristics of the BBB do not fully develop until the neonatal period. Because the BBB in fetal and early neonatal life is not fully developed, it allows for the diffusion of bloodborne macromolecules and toxins that are normally excluded from the mature CNS. Apparently, the fetus and neonate are at greater risk for brain injury from toxicants, such as CWAs, metals, pesticides, and other environmental contaminants.

48.6 Transport of molecules across the BBB

Brain capillary endothelial cells form the BBB. They are connected by extensive tight junctions and are polarized into luminal (blood-facing) and abluminal (brain-facing) plasma membrane domains (Hawkins et al., 2006). A pivotal function of the endothelial cells is to express transporters at the BBB and regulate the selective transport and metabolism of substances from blood to brain (Daneman et al., 2010). These transporters may be utilized to target specific molecules for delivery into the brain for therapeutic purposes. Fig. 48.2 depicts various transport mechanisms for amino acids, glucose, proteins, and other molecules.

There are two major pathways for molecules and cells to cross the BBB: the paracellular (junctional) route and the transendothelial route (Deli et al., 2005). One of the hallmarks of the BBB phenotype is the restrictive paracellular pathway, which is regulated by interendothelial tight junctions. Tight junctions not only restrict paracellular flux but also maintain the polarity of enzymes and receptors on luminal and abluminal membrane domains. The most important integral tight junction proteins include occludin, claudin-1, claudin-5, and junctional adhesion molecules. Daneman et al. (2010) identified several tight junction molecules whose expression was found at the BBB, including marveld2, cingulin-like-1, and pard3, that might play a crucial role in the formation of BBB tight junctions.

It is established that the brain environment signals brain endothelial cells to form the BBB, but the identity of these signals is unknown. Paracellular permeability is regulated by diverse signaling cascades (Krizbai and Deli, 2003). Among many signaling pathways, some are noteworthy, such as Wnt/β-catenin signaling (Leibner et al., 2008; Daneman et al., 2010), sonic hedgehog (Shh) signaling (Alvarez et al., 2011), intracellular stabilization signals mediated VE-cadherin (Rudini et al., 2008; Taddei et al., 2008), and retinoic acid signaling (Mizee et al., 2013). Daneman et al. (2010) generated a comprehensive data set describing the transcriptome of the BBB, which will provide a valuable resource for understanding the development and function of this crucial barrier, as well as its role in modulating CNS function.

The transendothelial pathways also exist at the brain microvasculature. In contrast to peripheral endothelium, the rate of pinocytosis is minimal, and free membrane diffusion applies mainly to small lipophilic molecules, for example, ethanol or nicotine (Pardridge, 2002). Daneman et al. (2010) utilized ingenuity pathway analysis (IPA) software to analyze the CNS endothelial enriched transcripts to identify signaling pathways that are enriched at the BBB or in peripheral endothelial cells. Various transport systems are present at the BBB to transport compounds in and out of the brain (de Boer et al., 1998). Active transport systems can be divided into three groups. First is carrier-mediated bidirectional transport, which is responsible for nutrient uptake in the brain. These transporters include glucose transporter GLUT-1, monocarboxylic acid transporter MCT1, large neutral amino acid transporter LAT1, or sodium-coupled nucleoside transporter CNT2 (and others). Second is efflux transport, which is unidirectional and delivers metabolites and xenobiotics from the brain to blood. Third is receptor-mediated transport by endocytosis and transcytosis, which is important for the brain supply of peptides and proteins, such as low-density lipoproteins, transferrin, leptin, and insulin (Pardridge, 2002). Bidirectional transporters include glucose transporter (GLUT-1), monocarboxylic acid transporter (MCT1), large neutral amino acid transporter (LAT1), or sodium-coupled nucleoside transporter (CNT2) (and others) (Pardridge, 2002). Pgp and MRP-1 multidrug resistance proteins, brain multidrug resistance

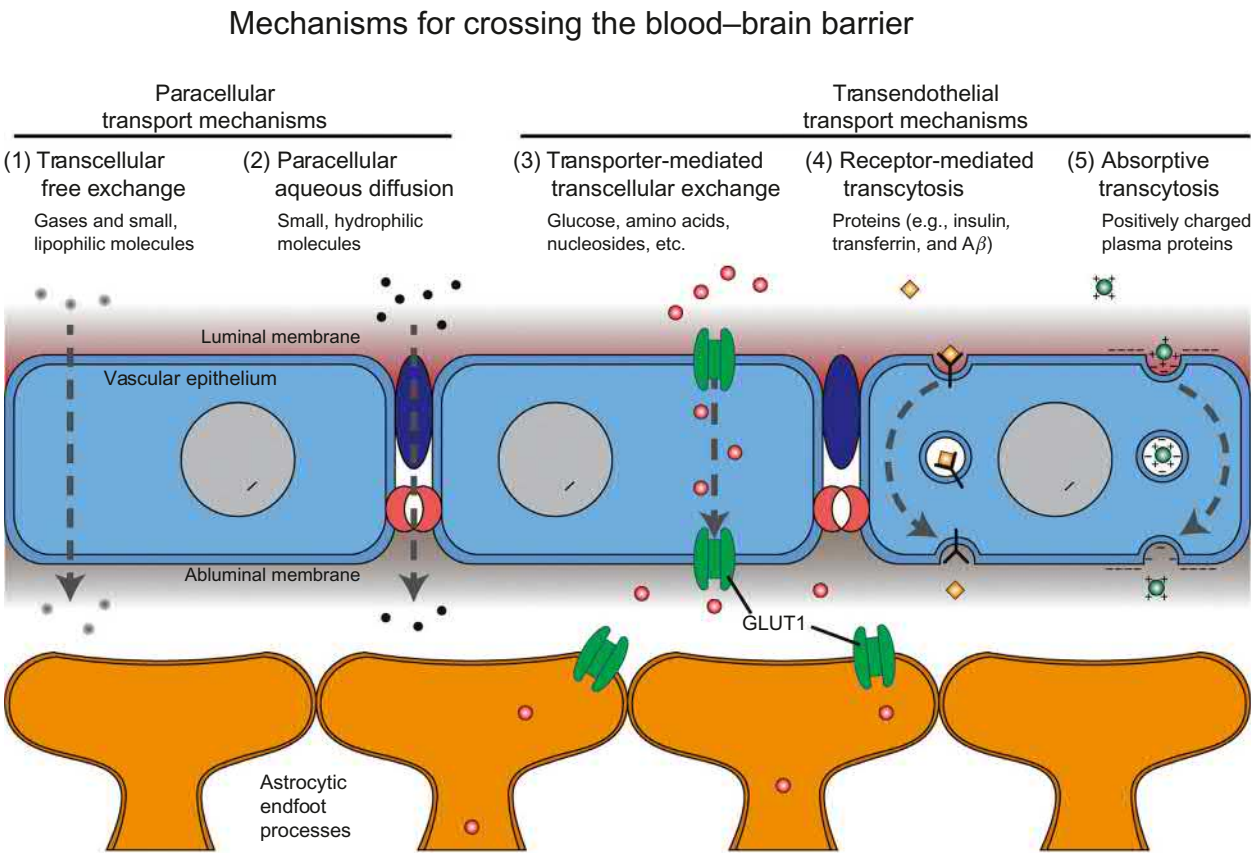

FIGURE 48.2 Transport across the BBB occurs through a variety of mechanisms.
Gases and small lipophilic molecules enter and exit the CNS freely through transcellular exchange. Very small hydrophilic molecules are able to permeate the tight junctions (dark blue ovals) and adherens junctions (salmon crescents) between vascular epithelial cells that restrict the entry of larger molecules into the CNS. Instead, larger hydrophilic molecules are transported through transmembrane pores, receptor-mediated transcytosis, or absorptive transcytosis. After crossing the BBB, solutes are then transported through the astrocytic endfoot processes and shuttled to neurons.

proteins (ABCG2/BCRP), or organic anion-transporting polypeptide (OATP2) belong to the rapidly growing group of efflux transporters at the BBB (de Boer et al., 2003; Chan et al., 2013). Additionally, ABCC1 and ABCC4 are expressed in the cerebral endothelium as efflux transporters (Wilhelm et al., 2011).

A number of cellular and molecular factors can influence transport of molecules across the BBB. For most solutes and macromolecules, permeability is largely dependent on their lipophilicity. Hydrophilic solutes and macromolecules are believed to cross the barrier through specific carrier mechanisms or facilitated diffusion (Aschner and Slicker, 1998). Some of these carriers are symmetrically distributed both on the luminal and abluminal membranes of the endothelial cells, whereas others have an asymmetric distribution. For example, the carriers for the essential neutral amino acids, which are required in the brain for neurotransmitter synthesis, are localized on both luminal and abluminal membranes. In contrast, the carrier for the amino acid glycine appears to be located only on the abluminal membrane. The function of this asymmetric distribution is to remove glycine from the CNS and to keep its concentration low in the brain. The polar distribution of proteins maintains amino acid homeostasis in the brain. The existence of two facilitative transporters for neutral amino acid on both membranes provides the brain with access to essential amino acids. It is now well-established that the BBB participates in the active regulation of the amino acid content of the brain. These comprise various amino acid transport systems (System L1, System ASC, System A, acidic amino acid transporter, peptide transporter) (Begley, 1995). In addition to these transporters, Pgp is one of the transporters of great interest (Wilhelm et al., 2011; Chan et al., 2013).

Daneman et al. (2010) and Geier et al. (2013) stated that the transporters and neuroprotective function of the BBB present major challenges for therapeutic drug delivery to the CNS. Critical to this function, BBB membrane transporters include the ATP-binding cassette (ABC) transporters, which limit drug penetration across the BBB, and the less studied solute carrier (SLC) transporters. These authors demonstrated expression of profiling of

359 SLC transporters and immunoassays in microvessels at the human BBB. In some situations, to be more effective, osmotic opening of the BBB has been used clinically to enhance entry of water-soluble drugs from the blood into the brain. Daneman et al. (2010) also asserted that IPA software can be used to identify metabolic pathways enriched at the BBB or in peripheral endothelial cells. Glycolysis/gluconeogenesis and amino acid metabolism are enriched at the BBB, suggesting that brain endothelial cells may be intimately involved in the production of energy metabolites and amino acids for neurons, a function that has been thought to be uniquely served by astrocytes (Bélanger et al., 2011).

48.7 Effects of toxic agents on the BBB

Breakdown of the BBB in humans and experimental animals in various conditions (stress, hypertension, convulsions, seizures, ischemia, hypoglycemia, inflammation) has been well-documented. The BBB is incapable of preventing the exchange of toxins/toxicants from the blood to the brain when its integrity (structure, function, or permeability) is compromised by chemicals or their toxicity.

48.7.1 Anticholinesterase organophosphate nerve agents

Drewes and Singh (1985) reported that the cerebral transendothelial carrier-mediated transport of glucose and amino acids was not affected in mongrel dogs poisoned by soman, an irreversible acetylcholinesterase (AChE) inhibitor. Carpentier et al. (1990) investigated acute changes in BBB permeability to proteins using EB-labeled serum albumin and plasmatic gamma-immunoglobulin G (IgG) as indicators in rats. An increased BBB permeability to the EB–albumin complex was macroscopically observed in two-thirds of the convulsive rats intoxicated by 85 μg/kg of soman. Soman produced seizures and reversible BBB opening to a greatest extent after 30–60 min of paroxysmal electroencephalographic (EEG) discharges when signs of cerebral hyperactivity (epileptic EEG pattern, hyperoxia) were also at their height. Topographically, the protein leakage was bilateral and restricted to anatomically defined brain structures, some of which were sites of parenchymal edema and neuronal damage. Vascular damage occurred approximately when toxic symptoms began to reduce and was nearly concomitant to the highest level of brain oxygenation and to the maximal intensity of seizures. Interestingly, the first signs of increased BBB permeability were shown to precede the onset of edema. Carpentier et al. (1990) detected the BBB opening in the amygdaloid complex and in some cortical regions (cingulum, entorhinal, and piriform complex). The thalamus was the most frequently and intensely affected structure, and the hippocampus remained free of exudated immunoreactive IgG.

Observations from various studies suggest that soman-induced brain alterations are predominantly related to seizures or brain hyperactivity or to a direct cytotoxic action of soman or acetylcholine (ACh) itself, or because of ChE inhibition (McDonough et al., 1987).

Domer et al. (1983) found increased permeability of the BBB by systemic administration of ACh. Of course, brain hyperactivity alone appears inadequate to be responsible for the BBB opening. Obviously, the short duration of the transient protein leakage (Carpentier et al., 1990) contrasted with the well-known long-lasting brain AChE inhibition induced by soman (Petrali et al., 1985). Several other anti-ChE compounds, such as physostigmine and paraoxon, are also known to produce the BBB opening for macromolecules that was seizure-dependent, reversible, and unrelated to brain ChE inhibition. Ashani and Catravas (1981) observed that in soman-intoxicated rats, induced damage to BBB integrity was significantly reduced, despite a high degree of AChE and BChE inhibition, and protected from seizures by nembutal or atropine. In essence, endothelial AChE or BChE plays no role in BBB opening, although it may function as an "enzymatic barrier" to ACh.

Although the exact mechanism is unknown, various contributing factors, such as increased electrical activity, oxidative/nitrosative stress, decreased energy supply and store, deleterious action of excitatory amino acids, enhanced calcium intrusion, and brain edema, seemed to play significant roles in the brain damage (Misulis et al., 1987; Carpentier et al., 1990; Solberg and Belkin, 1997; Gupta et al., 2001a,b; Zaja-Milatovic et al., 2009; Prager et al., 2013; Milatovic et al., 2019). Other mechanisms in soman-induced damage to BBB integrity may be related to vasoactive substances (ACh, amines, amino acids, peptides, free radicals, and steroid hormones of the pituitary adrenal axis) and vasogenic events (acidosis, increased blood flow, and hypertension).

OP nerve agents, which are small lipophilic molecules, can easily penetrate the BBB by free diffusion and thereby inhibit AChE in the CNS (Mercey et al., 2012). Increased BBB permeability by OP nerve agents or other ChE inhibitors may lead to their enhanced entry into the brain, resulting in greater AChE inhibition and possibly resulting in subsequent maintenance of seizures and aggravation of their pathological consequences, such as edema and neuronal loss in certain brain structures. Evidently, increased BBB permeability may facilitate the entry of an antidote (oxime class) to the brain, which otherwise has limited access because of the BBB.

48.7.2 Oxime reactivators of AChE inhibited by OPs and the BBB

AChE-reactivating oximes can be categorized into four groups: charged or noncharged and one-ring or two-ring compounds (Worek and Thiermann, 2013; Esposito et al., 2014). Commonly used AChE reactivators (2-PAM, MMB-4, and HI-6) against OP nerve agents/pesticide-induced AChE inhibition are permanently charged cationic compounds that do not appreciably cross the BBB (Melchers et al., 1994; Cassel et al., 1997; Mercey et al., 2012; Esposito et al., 2014). These oximes reactivate AChE in peripheral sites, but they are not effective in the CNS and, consequently, they provide little or no protection against the neurological/neurotoxic effects of OP nerve agents. However, BBB-penetrating uncharged oximes, such as monoisonitrosoacetone (MINA), diacetylmonoxime (DAM), and dihydropyridine 2-pralidoxime (pro-2-PAM), act centrally and abrogate brain OP-induced seizure activity. But these compounds have a much lower propensity for reactivation of ChEs in peripheral tissues and blood compared with 2-PAM and other quaternary oximes, and they are too toxic for use (Skovira et al., 2010; Demar et al., 2010).

Okuno et al. (2008) examined the BBB penetration of novel PAM-type oximes (alkylPAMs) using brain microdialysis with LC-MS/MS. Findings revealed that 4-[(hydroxyimino) methyl]-1-octylpyridinium bromide (4-PAO) may be effective for the reactivation of inhibited ChE in the brain; however, its toxicity was greater than that of 2-PAM. Recently, Okolotowicz et al. (2014) reported that uncharged amidine-oxime reactivator, (*z*)-*N*-((*E*)-1-(dimethylamino)-2-(hydroxyimino)ethylidine) butan-1-aminium chloride, is more lipophilic, chemically and metabolically stable, nontoxic, and can penetrate the BBB in animals and protect from the toxicity of nerve agents. Chambers et al. (2013) and Pringle et al (2018) evaluated phenoxyalkyl pyridinium oximes that are more lipophilic than currently approved oximes against OP nerve agent surrogates. Being more lipophilic, these oximes may penetrate the BBB easily and thereby reactivate the OP-inhibited AChE. Furthermore, Esposito et al. (2014) developed QSAR models to understand and improve physicochemical properties for the reactivation of OP-inactivated AChE, and these novel oximes may have easy access to cross the BBB as well.

Other strategies to deliver oximes across the BBB may include facilitative transport where sugar-oxime conjugates could be transferred by glucose transporters (Mercey et al., 2012; Bhonsle et al., 2013). Bhonsle et al. (2013) developed and validated molecular models for AChE reactivation by sugar oximes, which appear to be relatively nontoxic with a guinea pig LD_{50} of 1590 mg/kg. It is demonstrated that sugar oximes are relatively better reactivators of AChE inhibited by OPs compared with monoamine quaternary pralidoxime derivatives.

In OP nerve agent poisoning, a combination of AChE-reactivating oximes provides better therapeutic efficacy compared with an individual oxime (Kassa et al., 2010, 2011a,b). Also, oximes (pralidoxime, trimedoxime, obidoxime, HI-6, HLo-7) provide greater neuroprotection against OP nerve agents when given in combination with an antimuscarinic drug (e.g., atropine sulfate) and an anticonvulsant drug (e.g., diazepam). In such scenarios, oximes alone or in combination, with and without atropine sulfate/diazepam, need to be evaluated at the BBB for their mechanisms of transport.

Karasova et al. (2010) evaluated 30 AChE-reactivating oximes for BBB penetration using an in vitro model. Findings revealed that monoquaternary AChE reactivators were able to penetrate the BBB, and their molecular structure and molecular weight appeared to be the influencing factors for passive transport. Regarding the transport of bisquaternary oximes, the connecting linker plays a key role in penetrating the BBB, for example, short linkers tend to facilitate penetration. Furthermore, the location of the oxime group on the pyridine ring influences passive transport into the brain. The optimum position of the oxime group was found to be at position four (i.e., para).

In essence, novel oxime reactivators (alone or in combination) that are capable of crossing the BBB and efficiently reactivating ChEs within the CNS are of great interest for protection of military personnel and civilians from nerve agents and OP pesticides.

48.7.3 NMDAR antagonist memantine and the BBB

Currently, an *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonist memantine is widely used as a neuroprotectant in neurodegenerative diseases, but is more commonly used in moderate to severe AD. In a series of experimental studies, memantine has also been shown to protect animals from seizures, lethality, AChE inhibition, oxidative/nitrosative stress, high-energy phosphate depletion, cytotoxicity, and morphological alterations in the dendritic system of the brain against OP nerve agents or their surrogates (McLean et al., 1992; Gupta et al., 2007; Zaja-Milatovic et al., 2009; Stojiljković et al., 2019). To protect or reverse OP-induced effects in the brain, memantine has to cross the BBB. Yet, the exact mechanism by which memantine crosses the BBB remains unknown. Mehta et al. (2013) identified the putative transporter involved in memantine disposition in the brain in mice. The findings implicate the involvement of an organic cation transporter regulated by proton antiport mechanisms

in the transport of memantine across the mouse BBB, possibly the organic cation/carnitine transporter, OCTN1. Furthermore, memantine brain uptake was markedly reduced by various cationic transporter inhibitors (such as amantadine, quinine, tetraethylammonium, choline, and carnitine), suggesting the need for further investigation in patients using memantine and other medications (multidrug regimens). In the case of OP nerve agent poisonings, memantine is given in combination with atropine sulfate, which does not easily penetrate the BBB, and the interaction of memantine and atropine sulfate needs to be investigated at the BBB. Recently, memantine was also given in combination with HI-6 and diazepam in addition to atropine (Stojiljković et al., 2019), suggesting crossing of these antidotes through the BBB.

48.7.4 Drugs of abuse-induced BBB damage

Currently, a large number of drugs of abuse are consumed by civilians as well as military personnel around the world. Among these illicit drugs, cocaine, morphine, and amphetamines [methamphetamine (METH), amphetamine, and methcathinone] are most commonly used (Milatovic et al., 2019). Because of its small size and lipophilicity, METH readily crosses the BBB by nonspecific diffusion. METH can induce BBB dysfunction in rodents, particularly in the limbic region including the hippocampus (Bowyer and Ali, 2006; Martins et al., 2017). Recently, Multani et al. (2019) described BBB disruption using protein tracers, such as EB, iodine, and albumin immunohistochemistry of IgG1 in the cortex, hippocampus, thalamus, hypothalamus, cerebellum, and amygdala, and METH-induced neurotoxicity. The appearance of albumin immunoreactivity in the neuropil and leakage of serum albumin into the brain tissue had been observed as a consequence of METH-induced BBB breakdown, further leading to neuronal damage, myelin degeneration, reactive astrocytosis in the parietal and occipital cortices, extensive degeneration of pyramidal cells, and activation of microglia in the amygdala and hippocampus of rats (Sharma et al., 2007). It is suggested that, in addition to direct damage of monoaminergic nerve terminals, the deregulation of the BBB in these areas potentially contributes to widespread METH-induced neurotoxicity. METH directly damages dopaminergic and serotonergic nerve terminals, but also causes BBB dysfunction, which is thought to contribute to its neurotoxicity (Silva et al., 2010). Martins et al. (2013) provided mechanistic evidence that BBB breakdown was attributable to endothelial nitric oxide synthase (eNOS) activation and enhanced transcytosis. The neurotoxic effects of METH-induced BBB damage have been linked to hyperthermia, because METH causes dose-dependent temperature increases. Antioxidant H-290/51 pretreatment prevented hyperthermia, neuronal damage, myelin degradation, glial response, and leakage of serum albumin into brain tissue, establishing the role of free radicals in BBB damage and oxidative stress in METH neurotoxicity (Milatovic et al., 2019).

ElAli et al. (2012) reported that repeated administration of low-dose METH induces a transient parenchymal stress response, reflected by JNK1/2 and p38 MAPK activation, accompanied by the induction of proinflammatory cell adhesion molecules ICAM-1 and VCAM-1 in cerebral microvessels. Although tight junction proteins occludin and claudin-5 were expressed at high levels, a differential regulation of ABC transporters was observed after METH administration. The luminal ABCB1, which carries its substrates from the vessel into the blood, was upregulated, whereas the abluminal ABCC1, which carries its substrates from the vessel into the brain (Kilic et al., 2008), was downregulated in brain capillary cells (ElAli et al., 2012). These studies confirmed that METH modifies the structure of the BBB. JNK signaling has been shown to be involved in BBB breakdown associated with cerebral ischemia and subarachnoid hemorrhage. Urrutia et al. (2013) demonstrated that METH-induced changes in BBB integrity were attributable to JNK1/2-mediated activation of MMP-9 and laminin degradation. These effects were observed after acute exposure to METH at doses comparable with those used by consumers, for example, doses ingested typically in the range of 50–500 mg.

48.7.5 Metals

Metals, such as manganese (Mn), lead (Pb), and mercury (Hg), are transported on endogenous carriers, which otherwise function in the transport of essential macromolecules (Aschner and Slicker, 1998; Song et al., 2014). Mn binds readily to transferrin without displacing iron (Fe) in plasma. Brain areas (pallidum, thalamic nuclei, and substantia nigra) with high Mn levels differ from those with high levels of transferrin receptors (nucleus accumbens and caudate putamen), suggesting that perhaps these sites may accumulate Mn through neuronal transport. Like Fe, Mn-loaded transferrin is taken up by receptor-mediated endocytosis at the luminal membrane of brain capillaries.

Pb can enter the CNS by more than one mechanism. The transport of Pb may occur either via the exchange of $PbCO_3$ with an anion or via exchange of an anion–ternary complex of $PbCO_3$ with another anion. Kinetic studies with ^{203}Pb continuously infused intravenously into adult rats revealed that ^{203}Pb uptake into different brain regions was linear with time up to 4 h after infusion (Bradbury and Deane, 1993). In the absence of organic ligands for Pb, the metal readily entered the CNS. However, the presence of albumin, l-cysteine, or EDTA during the vascular

perfusion completely abolished the measurable uptake of ^{203}Pb. It was also suggested that Pb may passively enter the CNS in the form of $PbOH^+$.

It is well-established that Pb accumulates in the choroid plexus of humans as well as animals, suggesting that the choroid plexus is a primary target for Pb-induced neurotoxicity. Behl et al. (2009) demonstrated that exposure to Pb results in a significantly increased accumulation of intracellular amyloid-β ($A\beta_{1-40}$) in rat choroid plexus tissues in vivo and in immortalized choroidal epithelial Z310 cells in vitro. Several mechanisms may lead to an increased Aβ level at the BCSFB: a diminished expulsion of Aβ molecules from the plexus cells to the extracellular milieu; an increased uptake of Aβ from the CSF, blood, or both; an increased synthesis of Aβ; and/or a reduced metabolism or degradation of Aβ. Pb-induced inhibition of the production of LRP1 (a key intracellular Aβ transport protein in the choroid plexus) may be responsible for the accumulation of Aβ, and may be a major risk factor for AD.

Developing fetuses and neonates are most sensitive to methyl mercury (MeHg)-induced neurotoxicity, because MeHg is more readily transported across the immature BBB and because of its inhibitory effects on cell division. Aschner et al. (1990) demonstrated that MeHg conjugated to cysteine is transported across the BBB via the neutral amino acid transport L-system. It was reported that the structural similarity of the l-cysteine−MeHg conjugate with the structure of methionine suggested that, because of the broad specificity of the L-system, it should transport cysteine−MeHg conjugates efficiently across the BBB.

Copper (Cu) and Fe are trace minerals that are essential for normal brain function. They play important roles as catalysts, gene expression regulators, and second messengers. Monnot et al. (2011) and McCarthy and Kosman (2015) investigated how BBB and BCSFB regulated Cu transport and how Fe levels altered brain Cu homeostasis. The findings demonstrate that both the BBB and BCSFB contribute to maintaining a stable Cu homeostasis in the brain and CSF. Cu appears to enter the brain primarily via the BBB and is subsequently removed from the CSF by the BCSFB. Fe deficiency has a more profound effect on brain Cu levels than Fe overload. Fe deficiency increases Cu transport at the brain barriers and prompts Cu overload in the CNS. The BCSFB plays a key role in removing excess Cu from the CSF.

48.8 Bacterial toxin-induced BBB damage

Bacteria, their cell wall components, and their toxins can induce severe damage and dysfunction to the BBB (Deli et al., 2005). Lipopolysaccharide (LPS) is the primary endotoxin involved in inflammatory processes, sepsis, and multiorgan failure caused by Gram-negative bacteria like *Escherichia coli* (*E. coli*) or *Haemophilus influenzae*. LPS has been found to affect the CNS and damage the BBB by either direct or indirect means (Wispelwey et al., 1988; Shukla et al., 1996; Banks et al., 2015). Wispelwey et al. (1988) reviewed several studies dealing with LPS-induced damage to the BBB. In one study, intracarotid injections of *E. coli* LPS in rabbits caused diffuse breakdown of the BBB. In a second study, intracarotid LPS injections in rabbits were followed by an injection of colloidal Fe 4 h later. Electron microscopy of the brains of the LPS-treated animals revealed a large quantity of Fe within the endothelial cells, basement membrane, and glial process; however, in the saline controls, the Fe remained confined to the vascular lumen. In a third study, microscopic evaluation of brains from cats that had received intracisternal injection of *E. coli* LPS revealed evidence of profound inflammatory cell infiltration and microcirculatory impairment in both meningeal and cortical microvessels. Free radicals and nitric oxide (NO) appear to play a role in the LPS-induced increase in BBB permeability because stimulation with LPS not only produces superoxide radicals, but also enhances NO production. Minami et al. (1998) investigated the roles of NO and prostaglandins (PGs) in the development of damage to the BBB induced by LPS using NOS and cyclooxygenase (COX) inhibitors. These authors concluded that both the NO produced by NOS (especially by iNOS) and the PGs produced by COX participate in the LPS-induced increase of BBB permeability. In in vitro BBB models, LPS induced a concentration-dependent and time-dependent increase in monolayer permeability (de Vries et al., 1996; Gaillard et al., 2003). Interestingly, glial cells protected cerebral endothelial cells from LPS-mediated injury in a coculture model.

Pertussis toxin, a virulence factor of *Bordetella pertussis*, severely compromises the integrity of brain endothelial monolayers in a dose-dependent and time-dependent manner and is possibly mediated by the PKC pathway (Brückener et al., 2003).

Meningitis-causing bacteria interact with brain endothelium and can cross the BBB as live bacteria either transcellularly or paracellularly and subsequently multiply inside the CNS (Kim, 2008). *Bacillus anthracis*, the etiologic agent of anthrax, has been shown to penetrate the BBB in vivo, and expression of the anthrax toxins was essential for this transmigration (van Sorge et al., 2008). Anthrax toxins are known to cause BBB disruption, invasion, trafficking, and the development of meningitis during live bacterial infection (Ebrahimi et al., 2011).

48.9 GWI and the BBB

After the first Persian Gulf War, many soldiers reported a variety of symptoms designated as Gulf War illness

(GWI). The long-term symptoms include chronic fatigue, musculoskeletal pain, and cognitive–psychological disturbances, such as memory loss, confusion, inability to concentrate, mood swings, irritability, and somnolence. Among several factors, the use of pyridostigmine bromide (PB) pills, given to protect troops from the effects of AChE-inhibiting OP nerve agents (such as sarin, soman, tabun), and pesticides used during deployment, is highly likely (Amourette et al., 2009). PB is a reversible AChE-inhibiting carbamate that has been recommended by most military health services for prophylaxis against intoxication with irreversible AChE-inhibiting nerve agents. The toxic signs associated with PB are due to overstimulation of nicotinic and muscarinic receptors in the peripheral nervous system (PNS). Because of its quaternary amine structure, PB has limited access to the CNS. Therefore, no effects on the CNS are described at doses currently recommended, unless BBB permeability is compromised.

Stress can disrupt the BBB and thereby can increase the neurotoxicity induced by chemicals in many cerebral areas (Sharma and Dey, 1986). Therefore, involvement of stress during PB treatment may allow PB to enter the brain and produce inhibition of brain AChE activity. It has been hypothesized that combat stress combined with PB treatment may have induced central penetration of PB, leading successively to the following: brain AChE inhibition; stimulation of muscarinic ACh receptors; rapid induction of c-fos oncogene; and selective regulatory effects on the long-lasting activities of genes involved in ACh metabolism (Amourette et al., 2009). Experimental studies have shown an increase in BBB permeability and inhibition of brain AChE activity after exposure to a combination of stress and PB (Friedman et al., 1996). At high doses, PB crosses the BBB in the hypothalamus to induce cholinergic and noncholinergic changes in nonstressed mice (Ropp et al., 2008). It is also suggested that the neurological symptoms of GWI are linked to neurodegeneration in some discrete brain areas, as clearly demonstrated in rats subjected to daily restraint stress and combined exposure to a repellent (*N,N*-diethyl-*m*-toluamide; DEET), an insecticide (permethrin), and PB (Abdel-Rehman et al., 2004).

48.10 Effects of blasts on the BBB

In several military conflicts, the incidence of explosive blast-induced traumatic brain injury (TBI) has been increased substantially. BBB disruption associated with TBI results in brain edema and increased cerebrovascular permeability, both of which affect morbidity and mortality in patients with head injury (Ling et al., 2009; Hue et al., 2013; Shetty et al., 2014). Brain edema after TBI is thought to be initiated by BBB rupture, permitting the influx of protein-rich exudate through compromised endothelial tight junctions that may lead to delayed neuronal dysfunction and degeneration (Shlosberg et al., 2010). In TBI, elevation of glutamate results in neuronal death primarily because of NMDAR-mediated excitotoxicity (Zhou and Sheng, 2013). Hue et al. (2013), using an in vitro model, reported the impact of primary blast on the BBB. By multiple measures, the barrier function of an in vitro BBB model was disrupted after exposure to a range of controlled blast loading conditions. Transendothelial electrical resistance (TEER) decreased acutely in a dose-dependent manner that was most strongly correlated with impulse, as opposed to peak overpressure or duration. Significantly increased hydraulic conductivity and solute permeability after injury further confirmed acute alterations in BBB function. Compromised zonula occludens-1 (ZO-1) immunostaining identified a structural basis for BBB breakdown. After blast exposure, TEER remained significantly decreased 2 days after injury, followed by spontaneous recovery to preinjury control levels by day 3. A study conducted on breachers (a military and law enforcement population that is routinely and repeatedly exposed to low-level blasts) showed changes in serum brain biomarker levels (ubiquitin C-terminal hydrolase-L1, αII-spectrin breakdown product, and glial fibrillary acidic protein), neurocognitive performance, and self-reported symptoms, suggesting brain injury and possibly damage to the BBB (Tate et al., 2013).

48.11 Excitotoxicity, stress, and the BBB

Glutamate excitotoxicity has been linked to chronic neurodegenerative disorders, including AD, amyotrophic lateral sclerosis (ALS), MS, and Parkinson disease (PD), as well as in ischemia and TBI (Deli et al., 2005; Marmiroli and Cavaletti, 2012; Gupta and Gupta, 2019). Škultétyová et al. (1998) reported a stress-induced increase in BBB permeability in control and monosodium glutamate-treated rats. Glutamate administration in neonatal rats causes reversible changes in BBB permeability and known neurotoxic lesions. These investigators evaluated whether glutamate repeatedly administered to neonatal rats influences properties of the developing BBB with consequences on adult BBB function. In control rats, 30 min of immobilization stress resulted in increased endogenous albumin extravasation in the hypothalamus, hippocampus, brainstem, and cerebellum, but not in the cortex and striatum. Basal levels of albumin in adult glutamate-treated rats (4 mg monosodium glutamate/g body weight, intraperitoneal, five times during neonatal period) were significantly lower in the hypothalamus compared with that in controls. Stress-induced increase in albumin levels was lower in the brainstem, higher in the hypothalamus, and similar in other brain regions in

glutamate-treated rats in comparison with controls. In conclusion, short-lasting immobilization stress increased BBB permeability in some, but not all, brain regions. Glutamate treatment of neonatal rats resulted in low basal albumin levels in the hypothalamus but did not exert a pronounced influence on adult BBB function. BBB vulnerability in glutamate-treated rats during stress exposure was increased in the hypothalamus and decreased in the brainstem.

In in vitro studies, although no permeability change was found in the case of basolateral application on a bovine coculture (Gaillard et al., 1996), apical glutamate treatment increased the flux of 70 kDa FITC-dextran (Collard et al., 2002) and decreased TEER (Sharp et al., 2003) in human brain endothelial monolayers. These findings support that brain endothelial cells express functional glutamate receptors (Krizbai et al., 1998; Sharp et al., 2003).

48.12 Brain barriers and CNS diseases

Involvement of brain interfaces, such as BBB and BCSFB, has been implicated in various neurodegenerative diseases, such as AD (Deane and Zlokovic, 2007; Zisper et al., 2007; Agyare et al., 2013; Burgmans et al., 2013; Srinivasan et al., 2016), PD (Shaltiel-Karyo et al., 2013), motor neuron disease (Garbuzova-Davis et al., 2007), Huntington's disease (Drouin-Ouellet et al., 2015), and MS (Minagar and Alexander, 2003; van Horssen et al., 2007; Basivireddy et al., 2013). Modulation of the BBB permeability has also been reported in stroke, TBI, epilepsy (Abbott and Friedman, 2012; de Vries et al., 2012; Hue et al., 2013), autism, schizophrenia and other psychiatric disorders (Shalev et al., 2009; Palmer, 2010), GWI (Amourette et al., 2009), and edema, hypoxic-reoxygenation, or ischemic conditions (Deli et al., 2005; Kaur and Ling, 2008).

Impaired cognitive function and short-term memory are characteristic clinical features of AD. Underlying pathological features of this disease include neuronal and synaptic loss in the cerebral cortex as well as amyloid-β (Aβ)-containing diffuse and neuritic plaques (senile plaques), intraneuronal neurofibrillary tangles, and cerebral amyloid angiopathy (Zisper et al., 2007; Agyare et al., 2013). A prevailing hypothesis in the AD field was the amyloid cascade hypothesis that Aβ deposition in the CNS initiates a cascade of molecular events that cause neurodegeneration, leading to AD onset and progression. However, because of poor correlation between insoluble amyloid and cognitive impairment, increased in vitro toxicity of Aβ in the presence of antifibrillogenic agents and the synaptic localization of soluble oligomeric polymers, many experts now support the soluble Aβ oligomer cascade hypothesis (reviewed in Wilcox et al., 2011). Amyloid plaques may instead act as Aβ sinks, plaques that are surrounded by a halo of oligomers, which then attach to synapses and eventually cause synapse loss (Koffie et al., 2009). The loss of synapses is highly relevant to cognitive impairments, and is the best correlate with AD dementia severity (Terry et al., 1991).

Zisper et al. (2007) provided evidence that in advanced AD, plasma proteins like prothrombin can be found within the microvessel wall and surrounding neuropil, and that leakage of the BBB may be more common in patients with at least one APOE4 allele. Agyare et al. (2013) demonstrated that DutchAβ40 shows preferential accumulation in the BBB endothelial cells because of its inefficient blood-to-brain transcytosis. Consequently, DutchAβ40 establishes a permeation barrier in the BBB endothelium, prevents its own clearance from the brain, and promotes the formation of amyloid deposits in the cerebral microvessels. Burgmans et al. (2013) presented ample evidence for interplay between Aβ and BBB function in AD and reiterated that accumulation of the Aβ and disruption of the BBB can initiate cerebral microangiopathy, which has frequently been associated with vascular dementia. Although Aβ and BBB dysfunction have both been associated with AD and vascular dementia, respectively, they coexist in most demented patients. In fact, increasing evidence suggests that Aβ and BBB disruption may interact and facilitate each other in their effect on neurodegeneration. Erickson and Banks (2013) published a review of BBB dysfunction as a cause and consequence of AD. Paganetti et al. (2014) demonstrated that oral treatment with pirenzepine dose-dependently reduced brain Aβ level by its enhanced clearance in AβPPS1, $hA\beta PP_{st}$, and AβPP/PS1 transgenic mice by selective inhibition of muscarinic ACh receptors on endothelial cells of brain microvessels at the BBB.

It appears that the BBB does not play a major role in the etiology of PD, but its disruption may be beneficial in drug development. The neuropathological hallmarks of PD are progressive loss of dopaminergic neurons in the substantia nigra pars compacta accompanied by inclusions termed Lewy bodies and dystrophic Lewy neuritis in surviving neurons. The main constituent of the Lewy bodies is the α-synuclein protein. The etiology of these proteins is thought to involve major conformational changes leading to their misfolding, followed by production of β-sheet structures that have a strong tendency to aggregate into small oligomers and protofibrils that elongate into mature fibrils (Leong et al., 2009). One of the main obstacles in drug development is the inability of most drugs to pass across the BBB into the CNS. Mannitol, a nonmetabolized FDA-approved osmotic diuretic mediator, can also be used to open the BBB by producing osmotic shrinkage of the endothelial cells and mechanical separation of the tight junctions that form the BBB. Shaltiel-Karyo et al. (2013)

demonstrated that mannitol interferes with α-synuclein aggregation without exerting adverse effects, and suggested that mannitol administration in combination with other drugs could be a promising novel approach for treating PD or other brain-related diseases.

In neurologic disorders such as MS, epilepsy, capillary cerebral amyloid angiopathy, and AD, a profound dysfunction of the BBB is apparent (de Vries et al., 2012; Gupta and Gupta, 2019). In stroke and TBI, acute BBB dysfunction causes vasogenic edema with the danger of transtentorial herniation, and the effects of chronic BBB impairment are involved in neuroinflammatory disorders, such as MS, AD, and epilepsy (de Vries et al., 2012). Studies have led to the belief that BBB disruption represents an early event in MS lesion formation, preceding the massive infiltration of leukocytes (mainly T lymphocytes and monocyte-derived macrophages), leading to myelin degradation and nervous tissue destruction (Minagar and Alexander, 2003). In vitro and in vivo animal studies and patient tissue studies showed a significant involvement of the disruption of BBB integrity and function in MS pathology. Alterations not only involved the modulations of the tight junction but also included a reduced expression of the efflux pumps and the ABC transporters (Kooij et al., 2011).

48.13 Melatonin and the BBB

CWAs are known to generate excess free radicals and cause excitotoxicity and neuroinflammation. Melatonin (*N*-acetyl-5-methoxytryptamine), being an indirect antioxidant and free radical scavenger, can modulate and control oxidative stress. Melatonin is also involved in vasomotor control and adrenal function. It possesses antiexcitatory actions, regulates immune function and energy metabolism, and exerts antiinflammatory properties (Jumnongprakhon et al., 2016). Melatonin is highly lipophilic and, consequently, easily crosses cell membranes, including the BBB. Considering all these properties, melatonin could be an excellent and suitable candidate molecule to prevent CWA-induced tissue damage (Pita et al., 2013).

48.14 Concluding remarks and future directions

The CNS is composed of various interfaces, such as the BBB, the BCSFB, and the blood–spinal cord barrier (BSCB). Brain capillary endothelial cells form tight junctions and are referred to as the BBB. Currently, there are many in vivo and in vitro models to understand the mechanistic aspects of BBB functionality and permeability. Under physiological conditions, brain barriers protect the brain from pathogens, toxins, toxicants, proteins, and neurotransmitters. Disruption of the normal function of the BBB to circulating solutes and toxicants is usually the result of widened interendothelial junctions and/or alterations in one of the transporters localized within the BBB. Structure and function of the BBB and its permeability can be modulated by CWAs, toxins/toxicants, adverse conditions, and induced pathological conditions. Disruption of the BBB can also be the cause and/or consequence of neurodegenerative diseases, such as AD and MS. In future studies, novel in vitro and in vivo methods need to be developed to screen the compounds with properties of easy access to the BBB and those that can be used as therapeutic agents against CWAs.

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Chapter 49

The effects of organophosphates in the early stages of human skeletal muscle regeneration

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49.1 Introduction

Organophosphate (OP) poisoning may result in myopathy (Wecker et al., 1978; Preusser, 1967; Karalliedde et al., 2006; Abdollahi and Karami-Mohajeri, 2012) which is—when it occurs—one of the most serious chronic consequences of such poisoning. The final outcome of this condition depends on the one hand on the extent of muscle damage, and on the other on the efficiency of muscle regeneration. The first stage of OP poisoning leads to muscular hyperactivity and a combination of factors, including mechanical stress, Ca^{2+} overload, and alterations in oxidative processes in the muscle fibers (Mense et al., 2003; Karalliedde et al., 2006; Abdollahi and Karami-Mohajeri, 2012) leads to destruction of the muscle tissue. During sustained seizures and fasciculations, the flow of oxygen through the muscle is greatly increased at a time when the demand for ATP is greater than its rate of generation. Under such conditions, production of reactive oxygen species is greatly increased and exceeds the protective capacity of the cellular defense system so that their damaging effects result in muscle injury, including necrosis of muscle fibers (Ariens et al., 1969; Abdollahi and Karami-Mohajeri, 2012; Gupta et al., 2002; Dettbarn et al., 2001, 2006).

Necrosis, the extent of which depends on the level of muscle activity, is followed by infiltration with inflammatory cells, clearing the debris, and regeneration of muscle fibers (Ariens et al., 1969; Robertson et al., 1993; Cantini and Carraro, 1995; McLennan, 1996). Skeletal muscle has the capacity to alleviate its damage by quite efficient regeneration which, however, might be affected by the conditions imposed by OP poisoning. Therefore, it is important to know whether OPs interfere in any way with the mechanisms involved in the process of muscle regeneration. This aspect of OP poisoning has not been examined in detail in human muscle. Here we provide evidence that the mechanisms underlying early stages of human muscle regeneration are affected by OPs.

OP poisoning is a complex condition with various primary and secondary effects. Increased acetylcholine (ACh) concentration in the cholinergic synapses attributable to acetylcholinesterase (AChE) inhibition leads to altered signaling in these synapses, causing various pathological effects, including fasciculations, diarrhea, failure of respiratory muscles, excessive bronchosecretion, and cardiac arrhythmias (Abou-Donia et al., 2016; Alahakoon et al., 2018; Chhabria and Bhalla, 2016). Hypoxia that develops as a combination of impaired exchange of gases in the lungs and bradycardia is an especially threatening complication. Especially with high OP intake, typically found with suicide attempts (Alahakoon et al., 2018), poisoning is often complicated by respiratory failure combined with metabolic and respiratory acidosis (Emerson et al., 1999; Abou-Donia et al., 2016). These patients must be treated in intensive care units and often require mechanical ventilation (Alahakoon et al., 2018). Critically ill patients frequently develop skeletal muscle dysfunction (Bolton, 2005; de Letter et al., 2001; Friedrich et al., 2015) that often persists even after hospital discharge.

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There is now substantial evidence that inhibition of AChE is not the exclusive mechanism underlying a wide variety of adverse consequences of OP exposure described previously (Terry, 2012). It is known that cholinesterases (ChEs) are not the only targets of OPs and that various intracellular mechanisms are modified because of direct or indirect OP actions (Terry, 2012; Lima et al., 2013; Jett and Lein, 2006). For instance, DNA microarray analysis of MCF-7 cells treated with OP diazinon disclosed overwhelming modulation (upregulation or downregulation) of a wide range of genes (Mankame et al., 2006). Although still poorly understood, such noncholinergic OP actions might directly or indirectly modify functioning of the complex intracellular mechanisms and importantly contribute to the effects of OP poisoning. Indeed, a recent RNA-seq study in salmon fed chlorpyrifos-methyl in doses that are below the threshold for systemic toxicity due to AChE inhibition leads to marked alterations in the brain and liver transcriptome (Olsvik et al., 2019). Similarly, a marked effect on transcriptome was observed in cultured thyrocytes treated with chlorpyrifos (Porreca et al., 2016).

Skeletal muscle injury always activates the regeneration process (Bischoff, 1979) and insufficient muscle regeneration might directly contribute to the above-mentioned myopathies (Prelovsek et al., 2006). Muscle regeneration shares its basic features with embryonic myogenesis, although it also differs from it in some respects (Yin et al., 2013; Bentzinger et al., 2012; Charge and Rudnicki, 2004; Feige et al., 2018; Dumont et al., 2015). The regeneration process starts from the dormant mononucleated satellite cells located between the basal lamina and cell membrane of the adult skeletal muscle fiber (Mauro, 1961; Katz, 1961). One of the key steps in the process of muscle regeneration is myoblast proliferation, which decisively determines the mass of regenerated muscle tissue. Our investigations into the effects of OPs on muscle regeneration were therefore focused on these early precursors of muscle fibers.

Recently, we identified the neuropathy target esterase-related enzyme (NRE, PPLA7) as a novel OP target in human skeletal muscle. NRE is highly homologous to the neuronal neuropathy target esterase (NTE), which was originally identified as a target enzyme for highly toxic OPs that cause delayed paralyzing syndrome (Richardson et al., 2013). If targeted by OPs in vivo, NRE might be involved in OP poisoning-related myopathy.

All experiments were performed in the in vitro model in which the process of human muscle regeneration is genuinely reproduced. We describe various OP influences on the early precursors of muscle development and discuss their potential effects on muscle regeneration. Because AChE is the most thoroughly characterized target of OPs, we also approached the question of its role and expression in the cultured human (mononuclear) myoblasts. Diisopropylphosphorofluoridate (DFP) was used throughout our studies.

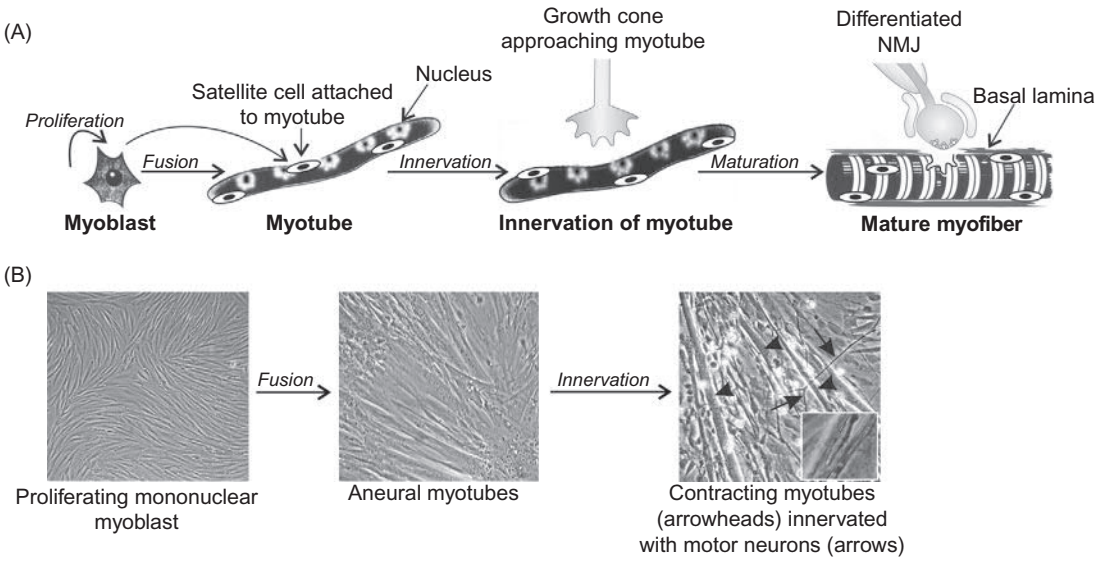

FIGURE 49.1 (A) Schematic presentation of the stages of muscle development. (B) Reproduction of these stages under in vitro conditions. (A) Stages of muscle development from mononuclear myoblast to the mature innervated myofiber. Nuclei, which are still centrally located in myotubes, move to the periphery in the mature fiber as a result of the synthesis of contractile elements in the sarcoplasm. Mature fibers contract and exhibit cross-striations. Only mononuclear myoblasts still have the capacity to divide and proliferate. Some of them do not fuse and become entrapped as mononuclear satellite cells under the basal lamina of the mature fiber. (B) Reproduction of this process in the experimental model of the in vitro innervated human muscle; axons are labeled by arrows and functionally innervated myotubes by arrowheads. Cross-striations could be seen in the innervated myotubes at higher magnification (insert).

49.2 Regeneration process in human skeletal muscle

The process of human muscle regeneration in many respects follows the process of embryonic development of skeletal muscle (Fig. 49.1A). The earliest myogenic precursors in this development are mononuclear myoblasts (Feige et al., 2018; Dumont et al., 2015; Yin et al., 2013; Charge and Rudnicki, 2004; Bentzinger et al., 2012). These still mononucleated, but already committed, cells proliferate several times to reach sufficient density before they fuse into multinucleated myotubes. Once formed, myotubes enter into a long-term and complex process of muscle differentiation that results in development of fully functional mature myofiber. An important step in the transition from myotube to myofiber is its innervation and formation of the complex structure of the neuromuscular junction (NMJ) (Emerson et al., 2004; Witzemann et al., 2013). All these steps are repeated during the postnatal regeneration process, except that adult mononuclear myoblasts are derived from the satellite cells. These primordial cells failed to enter the developmental and differentiation process during embryonic myogenesis, but retained the capacity to enter the myogenic process and self-renewal when activated by the muscle damage (Collins, 2006). As such, they serve as mononuclear muscle precursors of muscle fibers formed de novo during muscle regeneration (Fig. 49.1A).

All stages of muscle regeneration can be reproduced in the experimental model of the in vitro innervated human muscle (Fig. 49.1B). This system was first described in the 1980s (Kobayashi and Askanas, 1985; Askanas et al., 1987) and was further characterized in studies in various laboratories, including ours (Mis et al., 2017). In this experimental model, satellite cells are released by trypsinization from small pieces of adult human muscle routinely removed at orthopedic operations (Dolinar et al., 2018; Lojk et al., 2015). The experiments using human muscle cells were approved by the National Medical Ethics Committee of the Ministry of Health of the Republic of Slovenia (permit numbers 63/01/99, 71/05/12 and 0120-698/2017/4) and were conducted in accordance with the Declaration of Helsinki and Good Laboratory Practice regulations. Satellite cells released by trypsinization are the source of mononucleated myoblasts that then proliferate and, at a certain density, start to fuse and form multinucleated myotubes. To establish innervation, an explant of the rat embryonic spinal cord is placed on a monolayer of muscle cells (Mis et al., 2017). After 6–10 days of coculture, motor neurons functionally innervate myotubes, which then start contracting. Such cocultures are long-lived and contract for up to 6 months. The morphology of the NMJs closely resembles the NMJ in vivo (Askanas et al., 1987). Also, glial cells in the embryonic spinal cord explant recapitulate temporally regulated developmental steps observed in vivo (Mars et al., 2001). For details of preparation and various applications of the aneural and in vitro innervated model, see our recent review on this topic (Mis et al., 2017) and previous studies (Grubic et al., 1995; Mars et al., 2003; Jevsek et al., 2004; Pegan et al., 2010; Lojk et al., 2015, 2017; Rezonja et al., 2013, 2014; Mis et al., 2013; Gros et al., 2014; Dolinar et al., 2018).

49.3 Noncholinergic effects of DFP in regenerating human skeletal muscle

49.3.1 The effect of DFP on IL-6 secretion from myoblasts and myotubes

Cultured human myoblasts and myotubes used in our experiments constitutively secrete interleukin-6 (IL-6) (Prelovsek et al., 2006; Pirkmajer et al., 2010; Gros et al., 2014). IL-6 is a major cytokine released from the skeletal muscle during exercise as well as inflammation (Pal et al., 2014; Pedersen and Febbraio, 2012; Pedersen and Febbraio, 2008; Pedersen, 2019). We found that IL-6 secretion from cultured skeletal muscle cells is stimulated by the major proinflammatory agents, such as tumor necrosis factor (TNF-α) and endotoxin lipopolysaccharide, and suppressed by dexamethasone (Prelovsek et al., 2006; Pirkmajer et al., 2010). Because IL-6 promotes myoblast proliferation (Austin et al., 1992; Austin and Burgess, 1991; Cantini et al., 1995; de Letter et al., 2001; Baeza-Raja and Munoz-Canoves, 2004; Serrano et al., 2008), it is assumed that the physiological meaning of myoblast IL-6 secretion under such conditions is promotion of muscle regeneration, so that the myopathy attributable to septic conditions is, at least to some extent, compensated by muscle regeneration.

Critical illness is often complicated by so-called critical illness polyneuropathy (CIP) and myopathy (CIM) (Friedrich et al., 2015). As already mentioned, OP poisoning resulting in severe hypoxia often causes critical illness. Although the incidence of CIP/CIM in OP poisoning has not been established, it possibly overlaps with the so-called intermediary syndrome, which develops 24–96 h after OP poisoning and is characterized by muscle weakness (Hulse et al., 2014). Nevertheless, it can be expected that development of CIM is even more likely in the setting of OP poisoning because OPs directly lead to development of myopathy (Dettbarn et al., 2006), which includes necrosis of muscle fibers (De Reuck and Willems, 1975). IL-6 is thought to play an important role in CIM, although its precise function has not been established (Friedrich et al., 2015). It is therefore important to find out whether the OP intoxication affects IL-6 signaling and, consequently, muscle regeneration. The effects

of OP on IL-6 secretion from human myoblasts can be expected because it has already been reported that OPs drastically interfere with cytokine signaling in the mouse immune system (Alluwaimi and Hussein, 2007).

We show that IL-6 secretion from human myoblasts is significantly inhibited by DFP (Fig. 49.2). IL-6 secretion was decreased by 50% in myoblasts, which might significantly reduce the efficiency of the muscle regeneration process in damaged muscle. The mechanism underlying this effect of DFP remains to be established and is probably complex. In our culture medium, DFP is hydrolyzed quickly and becomes practically inactive in less than 1 h (Worek et al., 2004). Despite rapid hydrolysis, DFP-induced intracellular alterations may persist beyond the period during which DFP is still active. In our recent study, we observed a 53% decrease in IL-6 secretion from human myoblasts after treatment with 10^{-5} M tabun (Katalinic et al., 2013). Our experiments are in accordance with the study of Zabrodskii et al. (2012), who demonstrated reduced concentrations of blood IL-6 in rats exposed to 30 days of sublethal doses of sarin and methylparathion (Zabrodskii et al., 2012). Also, chlorpyrifos-oxon, a metabolite of chlorpyrifos, reduced IL-6 mRNA levels in mouse cortex and hippocampus (Locker et al., 2017). In contrast, DFP treatment in mice increased expression and/or secretion of TNF-α and/or IL-6 in the brain (Liang et al., 2018; Locker et al., 2017). In another mouse model, DFP increased IL-1β and IL-6 mRNA levels in the hippocampus, while TNF-α mRNA was not increased (Li et al., 2015). Taken together, these results indicate that the extent and pattern of cytokine response may vary across brain regions, cell types, and/or experimental approaches (Li et al., 2015; Locker et al., 2017; Liang et al., 2018).

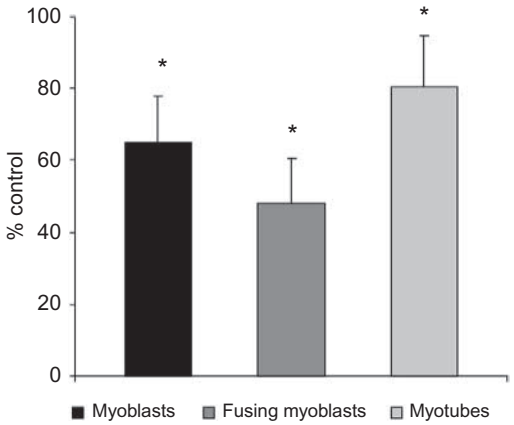

FIGURE 49.2 IL-6 secretion from myoblasts, fusing myoblasts, and myotubes. Cultured human myoblasts, fusing myoblasts, and myotubes were exposed to DFP (10^{-5} M). Concentration of IL-6 (expressed per 100,000 nuclei) was determined 24 h later with ELISA (Endogen, Rockford, IL). *Significant difference (Student t test, $P < .05$; $n = 3$) between control and treated cultures was observed at all stages studied, but was most prominent in the myoblasts.

49.3.2 Heat shock proteins in human myoblasts and myotubes after treatment with DFP

One of the most prominent cellular responses to stress is a rapid upregulation of heat shock proteins (Hsp), a family of highly conserved proteins that exert cytoprotective effects attributable to their chaperone functions in protein folding and protein degradation (Welch, 1992; Morimoto, 1993; Kregel, 2002; Kiang and Tsokos, 1998; Rosenzweig et al., 2019). Various physical, chemical, and biological environmental stress factors including xenobiotics can induce this response (Wu and Tanguay, 2006). Because OP pesticides are among inducers of the Hsp response in vivo and in vitro (Bagchi et al., 1996), we tested whether DFP actions include Hsp-mediated stress response in myogenic precursors. We measured the expression of Hsp27 and Hsp70, which are typically induced by various stress factors. The expression of stress factor Hsp27, but not of Hsp70, was slightly but significantly increased in DFP-treated human myoblasts (Fig. 49.3), which is consistent with the reported selectivity of Hsp induction in various tissues (Wu and Tanguay, 2006).

We observed the DFP-stimulated increase in Hsp70 expression in myotubes, which indicates that the Hsp response to DFP becomes more prominent during the myotube stage (Fig. 49.3). This pattern of effects is the opposite to the observed decrease in IL-6 secretion (Fig. 49.2), indicating that Hsp-mediated stress response does not promote IL-6 secretion. An increase in Hsp70 was also demonstrated in several organs of common carp exposed to chlorpyrifos (Xing et al., 2013), suggesting a

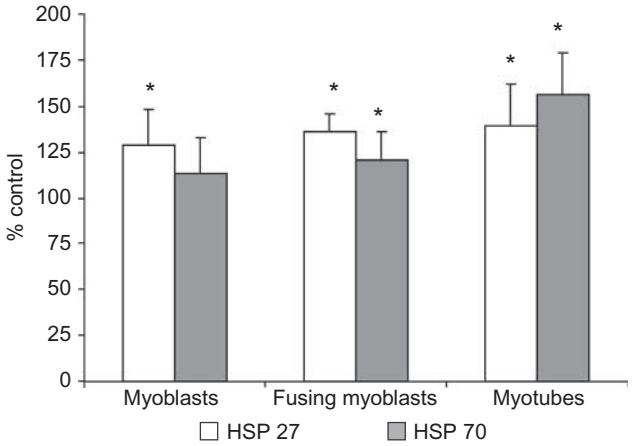

FIGURE 49.3 The effects of DFP on the Hsp27 and Hsp70 levels in myoblast, myoblasts in fusion, and myotube cultures. Levels of Hsp27 and Hsp70 were determined 24 h after addition of DFP (10^{-5} M). They were quantitated by western blot with Chemi Genius BioImaging System (Syngen, Cambridge, United Kingdom). *Significant difference (Student t test, $P < .05$; $n = 4$) between control and DFP-treated cultures was observed in all determinations except for Hsp70 in myoblasts.

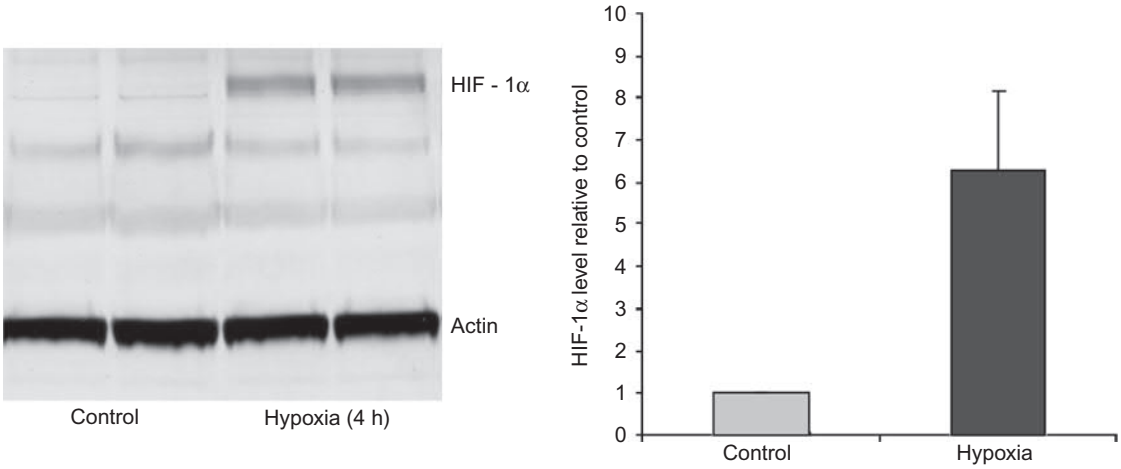

FIGURE 49.4 The effect of acute hypoxia on HIF-1α expression in human myoblasts. Human myoblasts were exposed to 1% O_2 for 4 h. A representative western blot is shown on the left. Relative expression level of HIF-1α (three independent experiments) is shown on the right (arbitrary units, control = 1) (Student *t* test, $P = .004$). Quantification was performed with Chemi Genius BioImaging System (Syngen, Cambridge, United Kingdom).

conservative mechanism unrelated to specific species, tissue, or OP. From the standpoint of muscle regeneration, it remains to be investigated whether the observed Hsp response to DFP in any way protects the proliferative potential of myoblasts that may be hampered by a DFP-induced decrease in IL-6 secretion. These results are again indicative of the wide spectrum of effects that OPs induce on the muscle tissue.

49.3.3 Response of human myoblasts to hypoxia

Severe OP poisoning may result in respiratory failure with arterial oxygen partial pressures less than 50−60 mmHg (6.65−7.99 kPa) (Tsao et al., 1990; Suzuki et al., 1997). In such circumstances, peripheral tissues, including skeletal muscle, become extremely hypoxic, which might have important effects on the myogenic precursors during regeneration. Central to the cellular response to hypoxia is hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor consisting of oxygen-regulated α-subunit (HIF-1α) and oxygen-independent β-subunit (HIF-1β) (Samanta and Semenza, 2017; Semenza and Wang, 1992; Semenza et al., 1991a, b). HIF-1α, which is normally almost undetectable because of its continuous degradation in the ubiquitin−proteasome pathway, rapidly accumulates in hypoxia and translocates to the nucleus, where it dimerizes with HIF-1β to form a functional transcription factor that controls the expression of hundreds of genes related to cellular adaptation to hypoxia (Samanta and Semenza, 2017; Semenza, 2007b).

Through its effects on gene expression, HIF-1α not only increases oxygen delivery to hypoxic tissues by stimulating erythropoiesis and angiogenesis, but also promotes cell survival by redirecting cellular energy metabolism toward glycolysis (Semenza, 2007a; Seagroves et al., 2001). Because of its central role in the hypoxic response, we tested whether such an adaptation also takes place in human myoblasts in vitro. We exposed human myoblasts to acute hypoxia (1% O_2 for 4 h) and found markedly increased HIF-1α levels compared with normoxic control (Fig. 49.4). This and our other results (Pirkmajer et al., 2010; Lojk et al., 2015) suggest that human myoblasts respond to hypoxia in a fashion similar to that of other cell types.

Myoblast proliferation is accompanied by apoptosis, which reduces the number of myogenic precursors and therefore affects the final outcome of the regeneration process. To determine if hypoxia affects muscle regeneration by altering the extent of myoblast apoptosis, the apoptotic markers were followed in human myoblasts under hypoxic conditions. We found that the activity of effector caspases in myoblasts remained unaltered during 48 h exposure to 1% oxygen, suggesting low susceptibility to hypoxia-induced apoptosis (Fig. 49.5A). Consistent with this notion, hypoxia did not lead to marked necrosis of myoblasts as assessed by measuring the activity of lactate dehydrogenase in the supernatant (Fig. 49.5B).

49.3.4 The effects of DFP on the NRE activity in human myoblasts

OP poisoning may lead to delayed polyneuropathy, which is thought to result from inhibition of neuropathy target esterase (NTE) (Glynn, 2006; Richardson et al., 2013; Johnson, 1969a,b). NTE-related esterase (NRE, PNPLA7), a *trans*-membrane serine esterase linked to the endoplasmic reticulum, has been identified as a member of the patatin domain-containing enzymes family (Kienesberger et al., 2008). Several isoforms of NRE

were detected in different stages of skeletal muscle regeneration in vitro (Fig. 49.6A). Notably, the 150 kDa isoform was markedly upregulated on fusion of myoblasts into myotubes, suggesting NRE might have a role in differentiation of myogenic precursors. NRE activity was decreased in human myoblasts exposed to DFP (Fig. 49.6B). Taken together, these results indicate that OPs may impair myogenesis via NRE inhibition. Inhibition of NRE may represent a novel mechanism underlying OP poisoning-related myopathy.

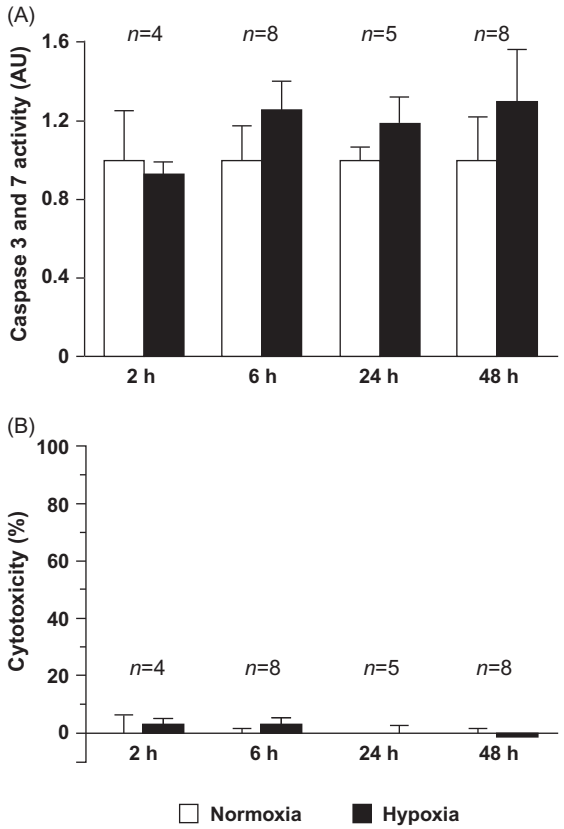

FIGURE 49.5 The effect of hypoxia on viability of human myoblasts. Cells were exposed to hypoxia (1% O_2) from 2 to 48 h as indicated. (A) Apoptosis and (B) cytotoxicity were estimated by measuring activity of caspases (3 and 7) and activity of lactate dehydrogenase, respectively. Results are means ± SD.

49.4 Expression and role of AChE in human myoblasts

Most of the contractile and synaptic muscle proteins cannot be detected before formation of myotubes. Conversely, AChE is already expressed in the myoblast stage (Tennyson et al., 1971; Grubic et al., 1995). Although its function in this earliest stage of muscle ontogenesis is not known, it is most likely noncholinergic or noncatalytic because other components of the cholinergic system are not present in myoblasts (Meshorer and Soreq, 2006). However, some studies suggested that skeletal muscle cells might be the source of ACh or a related cholinergic agent, whose function might be to stimulate nicotinic AChR in an autocrine and paracrine manner (Bandi et al., 2005; Hamann et al., 1995). Thus, the catalytic role of AChE in myoblasts and/or myotubes cannot be excluded. To further explore the role of AChE in myoblasts, we followed various functional parameters in human myoblasts after selective elimination of AChE expression by siRNA.

49.4.1 Recovery of AChE mRNA expression and AChE activity after gene silencing of AChE and after exposure to DFP

De novo AChE synthesis importantly contributes to the recovery of AChE activity after OP poisoning (Grubic et al., 1981). In order to find out whether OPs influence this synthesis at the mRNA level, and if they induce any morphological or functional changes of human myoblasts,

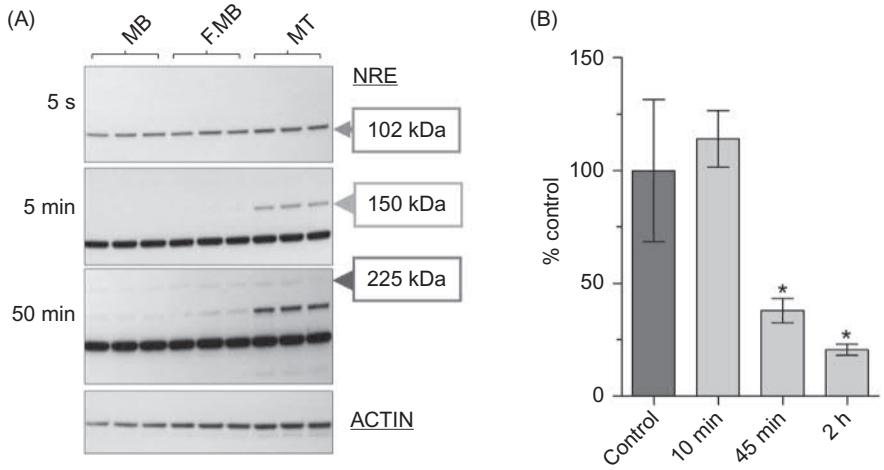

FIGURE 49.6 (A) Expression of NRE in cultured human skeletal muscle cells. (B) Inhibition of NRE by DFP. (A) Expression of NRE in cultured human myoblasts (MB), fusing myoblasts (F.MB), and myotubes (MT). Several immunoreactive bands were detected with the anti-NRE antibody at 102, 150, and 225 kDa (note different times of film exposure) with a significant increase of 150 kDa isoform between fusing myoblasts and myotubes. (B) Time-dependent inhibition of NRE-specific esterase activity by DFP. Myoblasts were exposed to 10^{-5} M DFP for 10, 45, and 120 min. NRE-specific esterase activity was measured in myoblast homogenates using *p*-nitrophenyl valerate as a substrate. Data shown represent means ± SE ($n = 4–6$). *$P \leq .05$ vs. control.

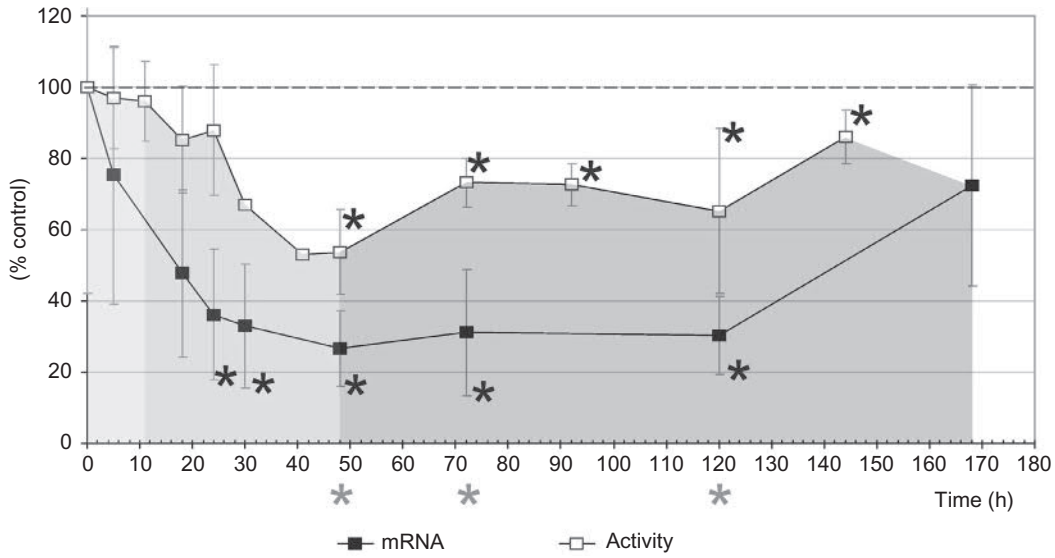

FIGURE 49.7 Relative changes of AChE-T mRNA and AChE activity in lysates of human myoblasts during the first week after siRNA application. Cultures of control and treated myoblasts were prepared from the satellite cells of the same donor and were processed in parallel. Gene silencing was achieved by lipofection of siRNA (Dharmacon); for details see Mis et al. (2006). Q-PCR with TaqMan chemistry was used for mRNA measurements. AChE mRNA levels were standardized to GAPDH mRNA. AChE activity levels determined by Ellman technique were expressed per 10,000 cells, the number of which was determined on the basis of Hoechst 33258 nuclear staining. Each point represents the mean (± SD) of three to seven measurements, and in each of which we determined the AChE-T mRNA or AChE activity ratio between control and siRNA-treated levels (% control). *The statistically significant (Student *t* test, $P < .05$) differences relative to the starting point. The statistically significant (Student *t* test, $P < .05$) percent differences between AChE activity and mRNA levels at the same time point are indicated by * under the *x*-axis. Three shades of gray correspond to the three stages discussed in the text.

we explored the recovery of AChE mRNA and AChE activity after gene silencing of AChE or after DFP exposure. AChE level (determined on the basis of its catalytic activity) decreased to approximately 50% of control after siRNA treatment (Fig. 49.7). No functional or morphological changes in myoblast cultures could be detected in comparison with controls. Addition of DFP to siRNA-treated myoblasts, which blocked practically all AChE catalytic activity, also resulted in no morphological changes in myoblast cultures. This observation suggests that complete loss of AChE catalytic activity exerts no visible effect in human myoblasts.

We then followed and compared the levels of AChE mRNA and AChE activity in siRNA-treated myoblasts and estimated the relationship between AChE expression at the mRNA and mature protein level. Because AChE is polymorphic and alternative splicing of primary transcript gives rise to three AChE mRNA species, tailed (T), hydrophobic (H), and read-through (R), which encode differently targeted AChE catalytic subunits (Massoulie, 2002), we followed these mRNA species separately in these experiments.

Three stages could be distinguished during a 170-h (approximately 7-day) period, during which we followed AChE activity:AChE mRNA ratio in the siRNA-treated myoblasts. During the first 10 h after siRNA treatment, AChE mRNA already decreased to approximately 60% of control level, whereas AChE activity remained practically unchanged. During the second stage (hours 10−50), AChE mRNA decreased to approximately 30% of control. AChE activity followed this decline and reached its lowest level of approximately 50% of control 50 h after siRNA treatment. From hour 50 onward, a new (more or less) constant relationship between AChE and its mRNA is established (Fig. 49.7).

Of the three mRNA species, AChE-H and AChE-T mRNA followed practically the same pattern of expression after siRNA treatment of human myoblasts, whereas AChE-R mRNA behaved in a completely different and less reproducible manner (Fig. 49.8). In comparison with the other two mRNA species, which decreased to approximately 20% of control after siRNA treatment, AChE-R mRNA never decreased to less than 50% of control. It quickly recovered and then increased so that 7 days after siRNA treatment, its level was almost 1.5 times higher than that in controls. After approximately 150 h, but not earlier, we also observed similar overshoot for AChE-H, but never for AChE-T. All three AChE mRNA species could be detected at all developmental stages. Patterns of their expression were developmental stage-dependent, but AChE-T was the predominant species in all stages.

At present, we have no explanation for the different expression pattern of R-mRNA species in comparison with the other two. Increased AChE-R mRNA levels have

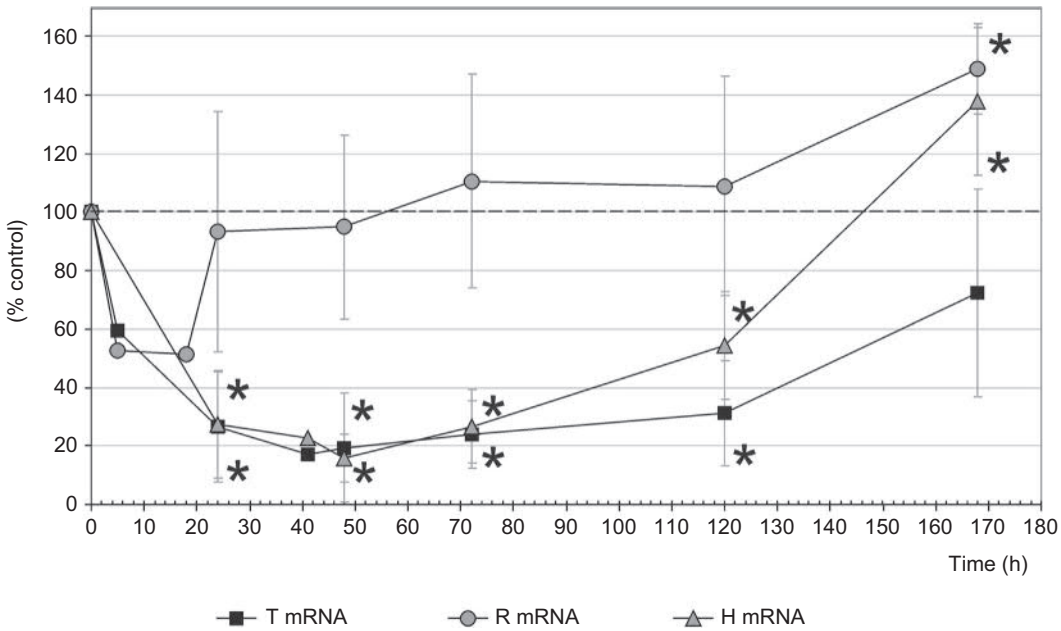

FIGURE 49.8 Relative changes of AChE-H, AChE-R, and AChE-T mRNAs in human myoblasts during the first week after siRNA application. Q-PCR with TaqMan chemistry was used for mRNA measurements. AChE mRNA levels were standardized to GAPDH mRNA. Each point represents the mean (± SD) of five separate siRNA experiments. In absolute terms, the levels of AChE-T mRNA were approximately 50 times higher than the levels of H and R. The differences vs. starting point (*) were statistically significant (Student *t* test, $P < .05$).

been reported in mouse brain after exposure to stress (Nijholt et al., 2004). However, under in vivo conditions, external factors not present in vitro could be responsible for changed expression of this mRNA. There have been several reports from the Soreq group in which AChE-R variant was specifically induced by various stressors (Cohen et al., 2003; Evron et al., 2007; Ofek et al., 2007; Grisaru et al., 2006; Shapira-Lichter et al., 2008; Shaltiel et al., 2013). However, we do not know whether our results obtained in an artificial environment in which systemic stress response is absent are related to these findings. In any case, R-species represent only a very small fraction of AChE mRNA, and it is not known whether it is translated. It might belong to the nontranslational RNAs, the role of which has recently been reported in various biological systems. No significant changes in the levels of any of the three AChE mRNA species could be observed at any of the developmental stages of human muscle after treatment with DFP, suggesting that OPs have no direct influence on AChE expression at the mRNA level in human muscle during the regeneration process.

49.4.2 The role of AChE in myoblast apoptosis

One of the frequently reported noncholinergic AChE functions is its participation in apoptosis. AChE was shown to be involved in regulation of apoptosis in different types of cells, including hematopoietic cells (Soreq et al., 1994), cultured smooth muscle cells, fibroblasts, endothelial cells and various cancer cells (Zhang et al., 2002), human neuroblastoma cells (Yang et al., 2002), and cultured human myoblasts (Pegan et al., 2010). Direct stimulation of apoptosis by AChE was noted in some cases (Toiber et al., 2008), but increased AChE expression per se is not necessarily sufficient to directly trigger apoptosis. Thus, AChE most likely promotes apoptosis primarily in conjunction with other apoptotic stimuli (Zhang and Greenberg, 2012), such as staurosporine (Pegan et al., 2010).

AChE may promote apoptosis by hydrolyzing ACh, which would tend to decrease prosurvival cholinergic signaling via nicotinic AChR and/or muscarinic AChR (Wessler and Kirkpatrick, 2008). Consistent with this notion, AChE inhibitors tacrine and physostigmine decreased apoptosis of fibroblasts (Zhang et al., 2002). In contrast, AChE inhibitors did not prevent apoptosis of Jurkat cells (Huang et al., 2005). Further, the C-terminal fragment of AChE (Day and Greenfield, 2004) as well as AChE that lacks cholinesterase activity both promote apoptosis (Du et al., 2015), indicating that the catalytic and proapoptotic activity are likely two separate functions of AChE. As for the molecular mechanism underlying these effects, there is evidence that AChE participates in apoptosome formation (Park et al., 2004, 2008) and that the AChE-T splice variant possesses DNase activity (Du et al., 2015). Inhibitors of AChE or mutations of the catalytic triad residues (S234A, E365A, and H478A) abolish

cholinesterase activity but not DNase activity of AChE (Du et al., 2015). Thus, pharmacological inhibitors of AChE may reduce apoptosis via conformational changes, which subsequently suppress proapoptotic activity of AChE, and not by inhibiting cholinesterase activity per se (Toiber et al., 2008). Taken together, while more detailed characterization of molecular mechanisms is clearly required, current evidence supports involvement of AChE in modulation of apoptosis (Zhang and Greenberg, 2012; Campoy et al., 2016).

AChE is expressed in proliferating myoblasts (Grubic et al., 1995), although these cells are deficient in most other components of the cholinergic system. To investigate if early AChE expression in myoblasts reflects its role in the development of the apoptotic apparatus, we followed the effect of siRNA-mediated AChE silencing on the apoptotic markers after staurosporine-induced apoptosis in cultured human myoblasts. We found that siRNA-mediated silencing of AChE in primary human myoblasts reduced staurosporine-induced activity of the initiator caspase 9 and the executioner caspase 3/7 (Pegan et al., 2010). AChE silencing reduced the fraction of apoptotic myoblasts as assessed with annexin V/propidium-iodide and 3,3-dihexyloxacarbocyanine iodide [DiOC6 (3)] staining, which confirmed AChE involvement in apoptosis of myoblasts (Pegan et al., 2010). In most cells which play no role in cholinergic transmission AChE is expressed only once these cells enter apoptosis (Zhang et al., 2002). In contrast, AChE is prominently expressed (Grubic et al., 1995) and active in proliferating myoblasts (Figs. 49.7 and 49.8) as well as in mature myofibers. Involvement of AChE in termination of neuromuscular transmission as well as apoptosis of myogenic precursors suggests these biological functions might be regulated by maintenance of different AChE pools. To regulate apoptosis, AChE should be localized in cytoplasm or nucleus, which is indeed the case in cultured myoblasts (Grubic et al., 1995). We may therefore speculate that in the apoptotic myoblasts AChE is diverted from the secretory route by which it is normally delivered to the extracellular space, to nucleus, cytoplasm, or other subcellular compartments, where it modulates apoptotic pathways. For instance, AChE may translocate into the nuclei of apoptotic cells and contribute to DNA degradation (Du et al., 2015).

49.5 Concluding remarks and future directions

Myopathies are a typical consequence of OP poisoning (Dettbarn et al., 2001, 2006). They might be additionally complicated by reduced efficiency of muscle regeneration. Our findings demonstrate that various intracellular mechanisms are influenced by exposure of precursors of muscle regeneration to DFP. Our experiments revealed reduced IL-6 secretion after DFP treatment, which might hamper myoblast proliferation and the efficiency of muscle regeneration. Increased levels of Hsps in DFP-treated myoblasts suggest that cellular response to stress is induced after DFP treatment. The increased level of HIF-1α after exposure to hypoxia shows that adaptation mechanisms against hypoxia are also organized during the early stages of muscle regeneration, while the apoptotic mechanisms in myoblasts apparently remained unchanged. We also demonstrated the role of AChE in apoptosis. As it has been demonstrated that AChE mutants lacking catalytic activity still participate in apoptosis it is possible that conformational changes of AChE induced by OP binding rather than inhibition of its catalytic activity are responsible for the OP effects on apoptosis. Whether such mechanisms affect muscle regeneration remains to be established.

Acknowledgments

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Chapter 50

Experimental modeling for delayed effects of organophosphates

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50.1 Introduction and background

Since the mid-20th century (Ellenbroek and Youn, 2016; Shimoyama et al., 2016), the rat, *Rattus norvegicus*, has remained one of the most popular species for laboratory studies. The central nervous system disturbances, renal and intestinal diseases, proteomics and metabolomics, sports medicine, effects of toxic and pharmacological preparations and nutraceuticals, are some of the scientific areas in which use of rats is indispensable (Barker-Haliski et al., 2017; Ellenbroek and Youn, 2016; Korf et al., 2017; Mindukshev et al., 2017; Singh et al., 2017; Sobolev et al., 2017; Ukolov et al., 2017). In toxicology, particularly in studying the action mechanisms of organophosphate (OP) toxic agents and in developing novel antidotal therapies, a major biochemical feature of rats that has to be considered is the presence of carboxylesterase (CE) activity in blood plasma. Mammalian carboxylesterases (EC 3.1.1.1) belong to a multigene superfamily with wide substrate specificity, being able to catalyze hydrolysis of esters, thioesters, amide-containing xenobiotics, and endogenous compounds, including fatty acid esters (Barker-Haliski et al., 2017; Hatfield et al., 2016). In contrast to rodents and lagomorphs, blood plasma of humans, monkeys, and hollow-horned artiodactyls does not contain CE (Lian et al., 2018). However, esterase activity is intrinsic to human serum albumin, the catalytic characteristics of which are far removed from those of rat and bovine serum albumins (Goncharov et al., 2015, 2017b; Li et al., 2005). Suppression of CE activity in rodent blood plasma can greatly increase the adequacy of experimental models in studying the action mechanisms of, and for developing novel antidotes against, highly toxic OPs such as soman, sarin, tabun, and paraoxon (Goncharov et al., 2017a; Maxwell et al., 1987; Maxwell, 1992). For these purposes, ES1 knockout mice (ES1−/−) are often used (Duysen et al., 2012). Knockout rats, however, have not yet been bred due to the complex and expensive technology involved. Use of specific CE inhibitors is a simpler and more reasonable approach, but has only been applied on a very limited scale. Consequently, the dynamics of long-term effects of acute OP poisoning against the background of suppressed rat plasma CE activity remained virtually unstudied. Moreover, even without preliminary suppression of CE activity, the consequences of acute OP poisoning have generally been studied over limited time intervals and using a limited list of indices (Duysen et al., 2011). Previously, we explored the dynamics of CE activity and some other biochemical and hematological blood indices at different times, from 3 h to 6 weeks, after acute soman or VX poisoning (Garnyuk et al., 2012; Flannery et al., 2016). The peculiarity of that series of experiments was injection of OP twice with a dose of $0.4LD_{50}$ at a 1-h interval. The first injection was aimed at partially suppressing rat plasma CE to allow the second injection to predominantly affect cholinesterases (ChE) in blood and synapses. A disadvantage of this model is the low selectivity of highly toxic OPs towards CE, especially considering that VX does not interact with CE at all (Maxwell, 1992). The aim of our recent studies was to carry out a comparative biochemical analysis of experimental models for investigating the long-term effects of acute OP poisoning with paraoxon (POX) and one of the selective CE inhibitors, 2-(*o*-crezyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide (CBDP) (Shmurak et al., 2012).

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50.2 Experimental procedures

Toxicological and biochemical studies were carried out on male outbred Wistar rats, *R. norvegicus*, weighing 200 − 240 g. Animals were kept in a vivarium at 20°C − 22°C, humidity ≤ 50%, and air exchange volumes (outdraught: inflow) of 8:10, under 12 h/12 h day/night light regime, in standard plastic cages with dust-free rat litter (PFLANZENFASER REHOFIX MK. 3500). Animals were given ad libitum access to water and standard lab chow pellets. Intact animals (negative control) received no injections and were kept in separate cages holding four rats each. Animals of a positive control were injected subcutaneously (s.c.) with a physiological solution instead of POX and kept in the same cage as their poisoned counterparts: a single positive control rat per three experimental ones. POX was injected to the withers' region (100 μL/100 g body weight) twice at a 1 h interval (POX2x, doses 0.45 + $0.6LD_{50}$), and in an hour after CBDP at a dose $0.6LD_{50}$ (CBPOX group). CBDP was injected intraperitoneally (i.p.) in the amount of 3.3 mg/kg. The indices were measure at 3, 24, and 72 h and at 1, 2, 4, 6, and 12 weeks after exposure. No less than six animals were taken for each time point.

Heparinized (50 IU/mL) blood was obtained after decapitation and centrifuged for 4 min at 1500 g. The plasma obtained was stored at −70°C until use. The following 27 biochemical markers were measured: whole-blood acetylcholinesterase (AChE), plasma cholinesterases (ChE), butyrylcholinesterase (BChE), plasma carboxylesterase (CE), paraoxonase 1 (PON1), alanine transaminase (ALT), albumin, total protein, glucose, D-3-hydroxybutyrate (3HB, beta-oxybutyric acid), triglycerides (TG), free fatty acids (FFA), glycerol, cholesterol, bile acids, high-density lipoproteins (HDL), low-density lipoproteins (LDL), lipase, iron, inorganic phosphate, alkaline phosphatase (ALP), urea, creatinine, calcium (Ca), uric acid (UA), amylase, and orosomucoid (alpha-1-acid glycoprotein, AAG).

Whole-blood AChE and plasma ChE activities were assayed by the Ellman method in its microplate modification (Prokofieva et al., 2010, 2012). Absorption was measured at 412 nm on a microplate spectrophotometer ThermoMultiscan FC (USA). Blood plasma biochemical analyses were carried out after defrosting on an automated biochemical analyzer Sapphire 400 (Japan) using commercial kits according to the manufacturer's recommendations. CE and PON1 activities were determined by nitrophenol output during paraoxon hydrolysis, according to Phuntuwate et al. (2005).

Software packages SciPy, NumPy, Pandas (McKinney, 2010; Pérez and Granger, 2007; Van Der Walt et al., 2011), Matplotlib, Seaborn, and Scikit-Posthocs (Hunter, 2007) were used, as well as the Kruskal-Wallis nonparametric test followed by the Conover test with Benjamini-Hochberg *p*-value corrections (Benjamini, and Hochberg, 1995). Data are presented as means ± SEM.

50.3 Toxicological data

The following basic POX toxicometric indices were established for male rats after a single s.c. injection: $LD_{16} = 241$, $LD_{50} = 250$, $LD_{84} = 259$ μg/kg. In a series of individual experiments we selected equitoxic POX and CBDP doses at 110 and 3.3 μg/kg, respectively. The objective was to select a CBDP dose that would not inhibit whole-blood AChE. It was established that 1 h after injection of POX (110 μg/kg) or CBDP (4 mg/kg) whole-blood AChE activity decreased more than twofold, while CBDP at 3.3 mg/kg and below did not inhibit AChE and BChE activities at all. After injections of POX (110 μg/kg + X) or CBDP + POX, repeated twice at a 1 h interval, the second POX dose required to achieve LD_{50} was established to be 165 μg/kg, that is, CBDP injection increased rat sensitivity to POX by approximately one third. This finding disagrees with other authors' data (Maxwell, 1992), which indicate that preliminary injection of rats with CBDP (2 mg/kg) increases sensitivity to POX twofold. The different method of CBDP injection, subcutaneous in previous experiments (Maxwell, 1992) or intraperitoneal in our hands, is the likely main cause of the differences in doses and the degree of sensitivity increase. A double POX poisoning of rats at doses of 110 and 150 μg/kg (0.45 and $0.6LD_{50}$; POX2x group) or POX injection at a dose of 150 μg/kg at 1 h after CBDP injection at a dose of 3.3 mg/kg (CBPOX group), did not lead to death of animals but induced clinical manifestations of cholinergic crisis, which is typical of the effect of nerve agents with the subsequent development of delayed pathologies. The latter is of great theoretical and practical value because the mechanisms underlying the development of consequences of acute poisoning are not yet clear.

50.4 Biochemical data

50.4.1 Cholinesterases

Among the above biochemical indices suitable for diagnostic purposes within the first hours and days after OP poisoning, the most sensitive is whole-blood AChE activity, which declined by almost an order of magnitude in both experimental groups by 3 h after poisoning (Fig. 50.1). It was 2 − 2.5-fold and 1.5 − 2-fold lower than in the control after 1 and 3 days, respectively, being restored within a week up to the negative control level but still remaining 20% − 30% lower than the positive

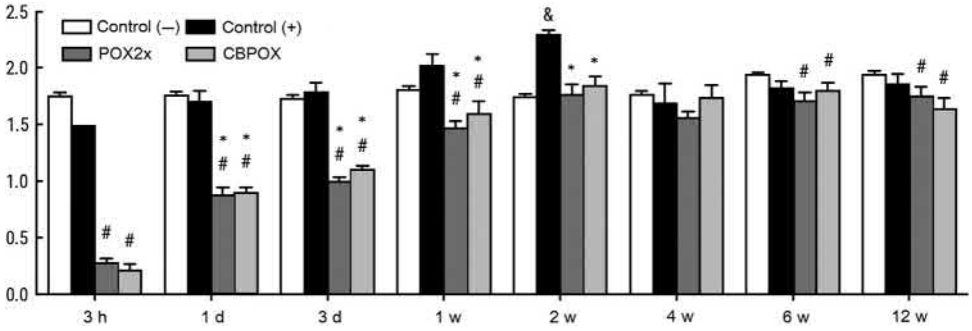

FIGURE 50.1 Activity dynamics of whole-blood acetylcholinesterase in control and experimental groups. *X*-axis (in this and other figures): time after poisoning, *Y*-axis: AChE activity (μmol/min/g Hb). Notes to Figs. 50.1 – 50.9: *, significant differences between experimental and positive control groups; #, significant differences between experimental and negative control groups; $, significant differences between experimental groups; &, significant differences between positive and negative control groups.

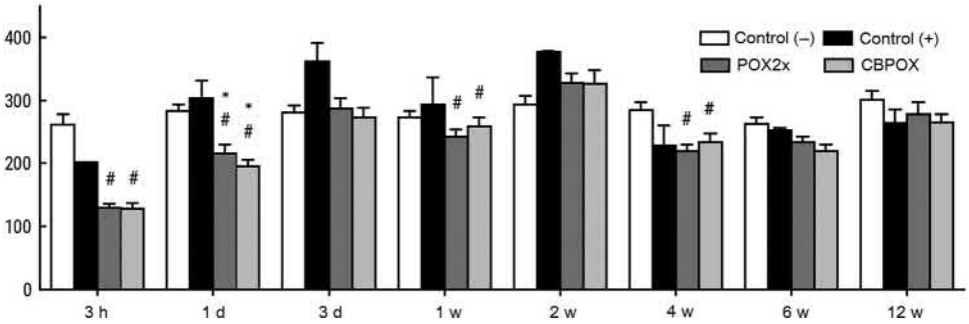

FIGURE 50.2 Activity dynamics of blood plasma butyrylcholinesterase in control and experimental groups. *Y*-axis: BChE activity (IU/L).

control level, which was slightly increased in activity compared to the negative control. The obtained data may indicate the influence of psychic stress in the positive control animals on the whole-blood AChE level. In this regard, it is worth mentioning a positive correlation described in the literature between human plasma ChE activities and human age, body mass index (BMI), stress level, inflammatory processes, and neurodegenerative diseases (Das, 2007; Dong et al., 2017; Reale et al., 2018; Shields et al., 2017; Sklan et al., 2004; Thayer and Sternberg, 2010). A complete restoration of AChE activity was registered at 2 weeks after poisoning, although increased AChE activity was still detected in the positive control group. This dynamic of AChE in the poisoned animals is more comparable with the changes we revealed after rat poisoning with soman (60% restoration in a week) than those with the V-type (VX) compound (complete restoration in a week) (Shmurak et al., 2012).

Both the amount and activity of plasma or serum BChE in rats are about 20-fold lower than in humans, and after OP poisoning the decrease was relatively low compared to that seen for AChE and restored completely by day 3 (Fig. 50.2). Total activity of rat plasma ChE was also decreased by twofold at 3 h after poisoning, but completely restored by the end of week 1; this could be explained by a higher rat plasma level of AChE compared to that of BChE (Goncharov et al., 2017a; Kurdyukov et al., 2012).

50.4.2 Carboxylesterase

Plasma CE activity decreased by three- and fourfold in POX2x and CBPOX groups, respectively (Fig. 50.3). Relative to the negative control, activity was restored by day 3, but in the positive control group it had increased by 15% – 20% in both the 1- and 3-day samples. Later, a steady trend was observed toward increasing level of CE activity in the positive control, POX2x and CBPOX groups, especially in 2-, 4-, and 6-week samples, reverting to the normal level in the 3-month sample. Differences were significant for each of the two experimental groups relative to the negative control after 2 weeks, but after 4 weeks a significant difference was only revealed for the POX2x group; after 6 weeks statistically significant deviations from the negative control were observed not only for experimental groups but also for the positive control group (Fig. 50.3). A foremost explanation of these findings could be that CE is one of the acute-phase proteins; to prove or reject this, one should study expression of the respective ES1 gene. An alternative explanation

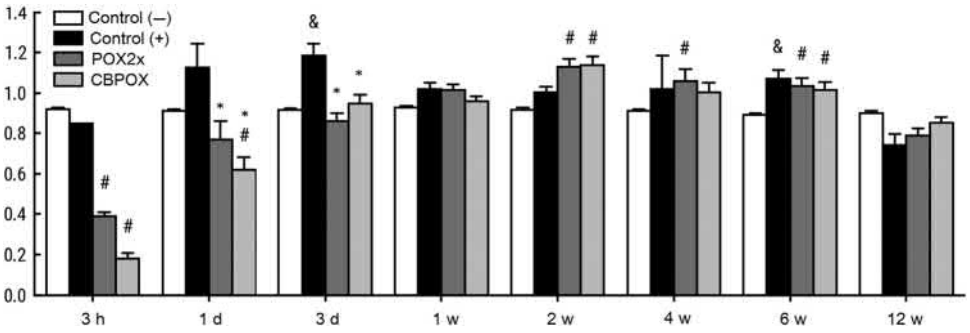

FIGURE 50.3 Activity dynamics of blood plasma carboxylesterase in control and experimental groups. *Y*-axis: CE activity (μmol NPA/min/mL).

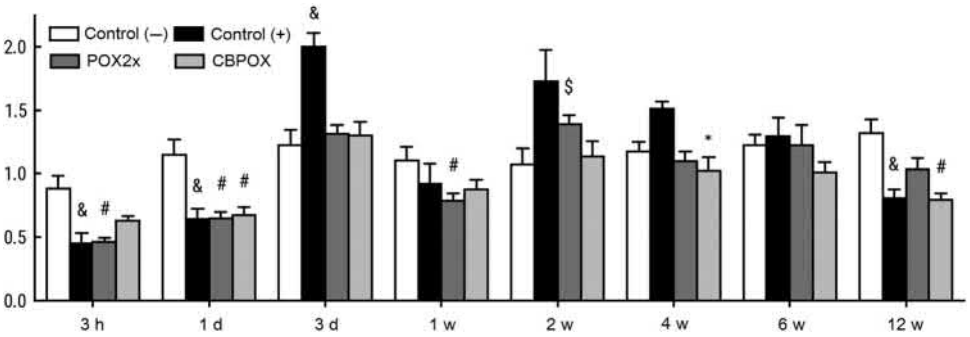

FIGURE 50.4 Activity dynamics of blood plasma level of triglycerides in control and experimental groups. *Y*-axis: TG concentration (mmol/L).

could be an affliction of the vascular endothelium and/or liver, where CES1 gene-encoded CE abounds along with the known tissue-specific markers (Orcholski et al., 2017). PON1 exhibited increased activity in 3-day, 2- and 4-week samples, and decreased activity 6 weeks after the poisoning, but only compared to the negative control group since measurements in the positive control group showed considerable deviations from the background activity unidirectional with those of the experimental groups. PON1 is a component of HDL, the level of which was increased in the intoxicated animals in 3-h and 1-day samples relative to the negative control, and decreased in both control groups by 3 days and 3 months after the poisoning. Moreover, the experimental groups exhibited an increased LDL level in the 3-month sample ($P < .01$).

50.4.3 Carbohydrate and fat metabolism

The indices of carbohydrate and fat metabolism were found to change at different time points after the poisoning, not only in experimental groups but also in the positive control group, with phasic changes evident for some indices. For example, at 3 h and 1 day, the TG levels were decreased almost twofold in three of the groups relative to intact animals; by day 3, it was restored in the poisoned animals, and exceeded the initial level 1.5 − 2-fold in the positive control group; then, the TG level showed some decrease by week 1 followed by an increase by week 2 and almost complete restoration by week 4 after poisoning (Fig. 50.4). The FFA levels also underwent phasic changes, which were most pronounced in the CBPOX group: an increase in 3 h, a decrease in 1 and 3 days, restoration in 1 week, a repeated decrease in 2 weeks, and the final restoration 4 weeks after the poisoning. Phasic changes in the glycerol levels were more pronounced in the CBPOX and, strange as it may seem, the positive control groups: an increase in 3 h and decrease in 1 day, then again, an increase in 3 days and decrease in 1 week, and the final return to initial level in 2 weeks. However, the 3HB dynamics was only demonstrated by the groups of poisoned animals: a three- to fourfold increase after 3 h, decrease down to the control level in 1 day, the second increase two- to threefold in 3 days, the second return to control level in 1 week, and yet another increase in 6 weeks, but only in the CBPOX group. Since 3HB is the most specific indicator of ketosis, compared to acetone and acetoacetate, it is reasonable to assume that CBPOX animals (to a greater extent than POX2x ones) are characterized by impaired glucose utilization, increased gluconeogenesis from proteins and fatty acids, reduced muscle mass, and increased risk of developing the metabolic syndrome. In 3 h after the poisoning, α-amylase increased almost equally in the POX2x and CBPOX groups (by 20% − 30%), however, its activity tended to increase more explicitly in the CBPOX group, indicating greater pancreatic damage in the toxicogenic phase.

Phasic changes in the cholesterol level in the positive control group were nearly identical to those in the POX2x group, with some differences: the maximum for the positive control fell on the first day, while that for the POX2x group was on the third day; and the amplitude of fluctuations in the POX2x group was more pronounced, within a minimum of a week after the poisoning. The levels of cholesterol in these groups came to the level of the negative control in just 4 weeks. The dynamics of cholesterol phasic changes in the CBPOX group was not as explicit, though it should be mentioned that the maximum fell on the third hour and the minimum on the sixth week after the poisoning ($P < .05$)! A decreased cholesterol level is known to be as harmful for an organism as an elevated one, being a risk factor for hemorrhagic stroke and unfavorable outcome of cardiac infarction and ischemic stroke (Iso et al., 1989; Markaki et al., 2014; Suzuki et al., 2011; Vauthey et al., 2000; Zhao et al., 2016; Zhou et al., 2016); moreover, a very low cholesterol level can promote the development of neurodegenerative diseases (Ferris et al., 2017; Fukui et al., 2015). The revealed metabolic disorders do not rule out that OP poisoning can lead to a delayed development of the hypometabolic status, which may manifest itself as neurodegenerative disorders, such as Alzheimer's disease (Landin et al., 1993).

50.4.4 Liver and kidney damage

The signs of liver damage were manifested most distinctly in the CBPOX group: a twofold increase in the ALT level in 3 h and 1 day after the poisoning (Fig. 50.5). In the POX2x group, changes were less pronounced: ALT increased by 20% − 30% at 3 h and twofold at 1 day. A complete reduction of the ALT level in POX2x and CBPOX groups occurred in a week, but in 4 weeks the ALT level had increased again by 10% − 20% in the poisoned animals (Fig. 50.5). Albumin and total protein were reduced insignificantly (by 5% − 10%) in 3 h, but a positive acute-phase protein AAG (orosomucoid) was increased almost threefold at 1 day after the poisoning (Fig. 50.6), reflecting a development of inflammatory processes. It should be pointed out that later the AAG level tended to increase relative to the negative control not only in the intoxicated rats, but also in the positive control group. Moreover, at some time points (1 and 2 weeks) this AAG increase in the positive control group was even more pronounced than that in the poisoned rats, reverting to the normal level in 3 months.

Kidney damage is indicated by the creatinine, urea, and uric acid biochemical indices. In the CBPOX group, the creatinine level was elevated by 35% − 40% at 3 h ($P < .05$) relative to both controls, while the urea level was increased by 20% − 30% at 1 and 2 weeks ($P < .05$), and relative to the negative control only. Previously, we detected very similar elevations of the creatinine level at 4 and 6 weeks after soman poisoning, and of the urea level at 4 and 6 weeks after poisoning with RVX and soman, respectively (Shmurak et al., 2012). Uric acid was one of the few indices for which the level changed significantly in our experiments exclusively in the POX2x group, by 20% − 40% at 1 day and 1 week. Since up to 80% of UA are excreted by the kidneys, this index provides additional evidence of kidney damage after acute OP poisoning. Besides, we detected an almost synchronous elevation in the levels of calcium ions and inorganic phosphate, as well as of ALP activity in rats of the CBPOX group in 3 h after the poisoning (Figs. 50.7 and 50.8). A simultaneous elevation in calcium and phosphate levels indicates intensified catabolic processes in tissues, including those under ketoacidosis, and renal dysfunction (Marshall et al., 2016). Considering increased creatinine, urea, and UA in blood plasma and increased glycosaminoglycans in urine (Sobolev et al., 2017), we view kidney damage as an integral component of cholinergic crisis consequences. Of particular note is a decrease in ALP activity that occurred 3 months after the poisoning for the three groups relative to the negative control, alongside an increase in the calcium levels in the same groups (Fig. 50.9). A combination of these characters may indicate hypomineralization of skeletal bones and even a development of the epileptic status (Buchet et al., 2013), as supported by the data from neurophysiological and

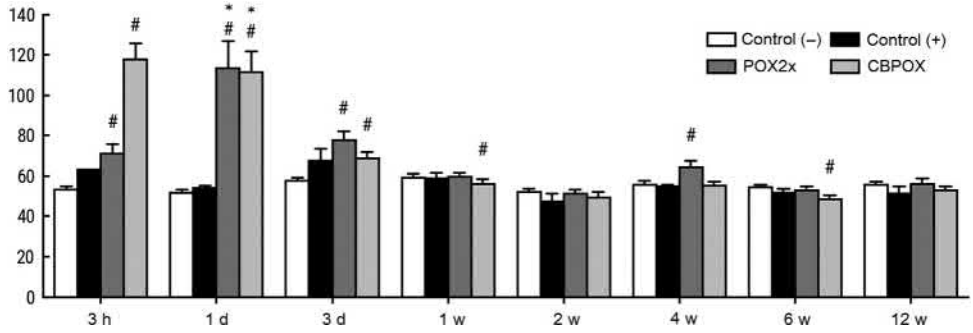

FIGURE 50.5 Activity dynamics of blood plasma alanine transaminase in control and experimental groups. *Y*-axis: ALT activity (IU/L).

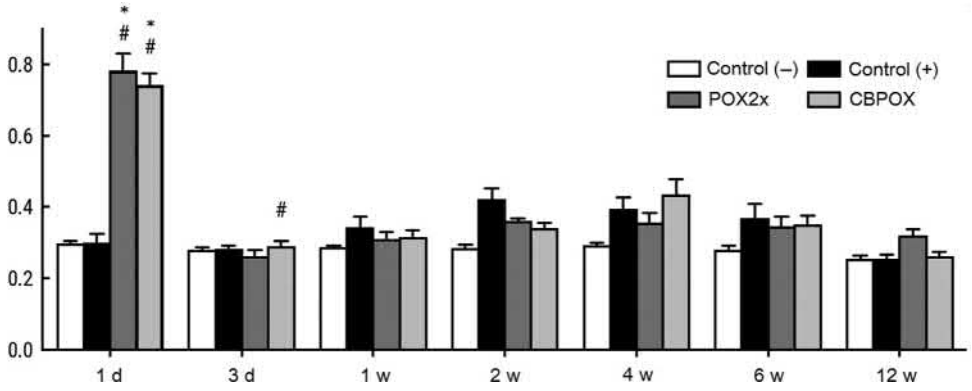

FIGURE 50.6 Activity dynamics of blood plasma alpha-1-acid glycoprotein (orosomucoid) level in control and experimental groups. *Y*-axis: orosomucoid content (g/L).

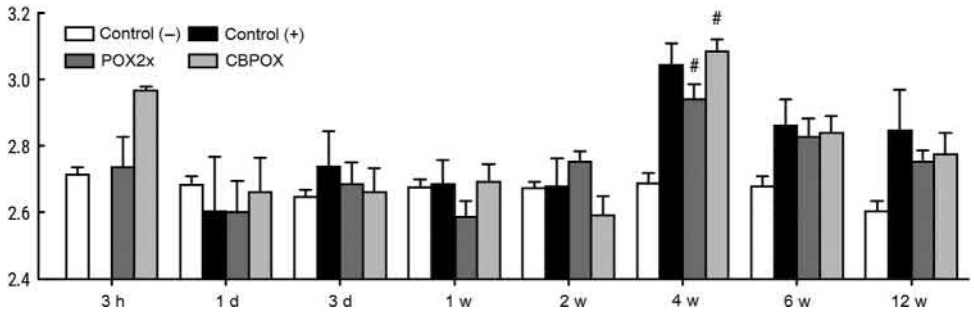

FIGURE 50.7 Activity dynamics of blood plasma concentration of calcium ions in control and experimental groups. *Y*-axis: Ca^{2+} concentration (mmol/L).

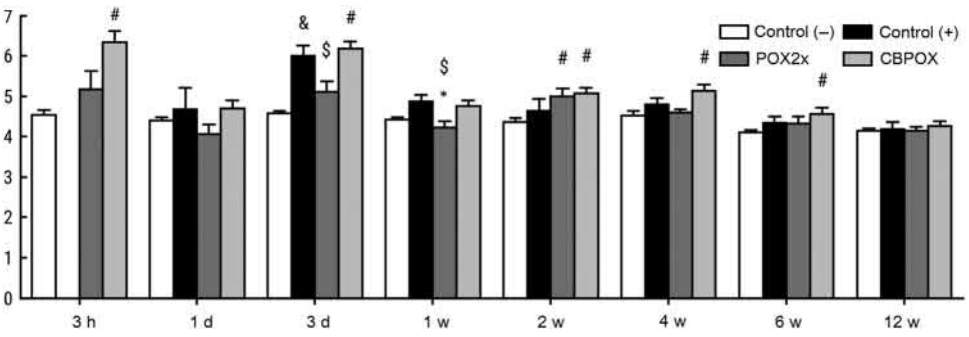

FIGURE 50.8 Activity dynamics of blood plasma phosphorous concentration in control and experimental groups. *Y*-axis: phosphorous concentration (mmol/L).

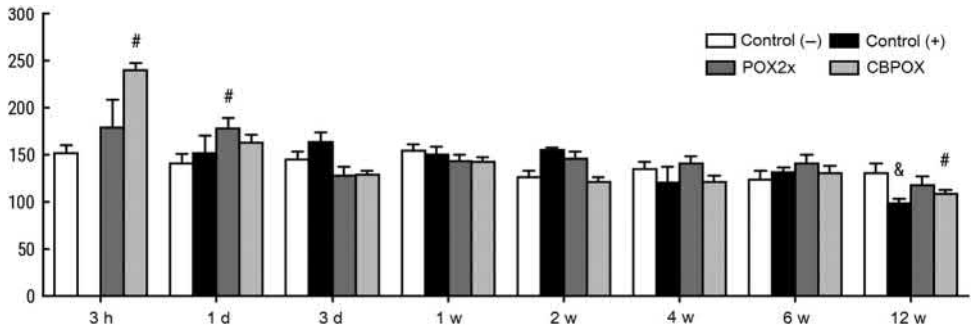

FIGURE 50.9 Activity dynamics of blood plasma alkaline phosphatase (ALP) in control and experimental groups. *Y*-axis: ALP activity (IU/L).

toxicological studies (Deshpande et al., 2016a,b). In addition, a reduced ALP hydrolytic activity relative to ATP, a physiological P2X7 receptor agonist, determines enhanced activation of these receptors and, as a result, decreased axonal growth rate (Sebastián-Serrano et al., 2014).

50.5 Concluding remarks and future directions

An important outcome of this study is the detection of statistically significant changes in a numerous number of biochemical indices in the positive control rats, which were permanently associated with the poisoned rats, relative to the negative control rats that were kept in isolated cages. Maximal differences were established in the dynamics of fat metabolism: a decreased TG level at 3 and 24 h was, nonetheless, elevated by 1.5 − 2-fold at 3 days and 2 weeks after the poisoning (Fig. 50.4). Elevation of cholesterol by 20% was evident in 1 day, while a reduction to the negative control level occurred in 4 weeks. However, the FFA level, being reduced in the positive control rats in 1 day after the poisoning by about 20%, later did not undergo any considerable changes relative to the negative control group.

We noted an increase in the AAG and a decrease in the albumin levels in the positive control rats 3 h after the poisoning. Interestingly, in these rats, maximally elevated BChE and AChE whole-blood levels also fell on the second week (Figs. 50.1 and 50.2). Blood plasma and hence whole blood in rats and mice, in contrast to human blood plasma, contains an appreciable amount of AChE [ca. 200 μg/L (Li et al., 2005)], with the plasma BChE level in rats being lower than that of AChE. As mentioned above and in a number of publications, plasma BChE and AChE were shown to be markers of low-level inflammatory processes (Das, 2007). It is also worth noting that there is a correlation between increased BChE activity, TG level, and decreased insulin sensitivity in patients with type 2 diabetes mellitus (Abbott et al., 1993). Our present data suggest a considerable metabolic impairment in the positive control rats that occurs within 2 weeks after the poisoning, evidently due to the stressful conditions. An analogous phenomenon has been reported in a study by Flannery et al. (2016), namely the development of neuroinflammatory processes in the CNS and the attenuation of cognitive functions in rats after acute OP poisoning; notably, these pathologies were expressed more or less equally in animals both with maximal and minimal manifestations of intoxication. Large-scale changes in the positive control rats relative to the negative controls are presented here for the first time. It appears that the state of anxiety and stress in rats is powerful enough to generate an additive or synergic effect with regard to numerous physiological and biochemical indices. This phenomenon deserves focused investigation in the future.

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Chapter 51

Alternative animal toxicity testing of chemical warfare agents

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51.1 Introduction

Chemical warfare agents have been in existence for centuries. They are substances which have toxic properties used to kill, injure, or incapacitate humans. Broadly, they are categorized into two groups: chemical or biological.

1. Chemical warfare agents use poisons that kill, injure, or incapacitate. These can be gases or liquids and are more commonly dispersed as aerosols.
2. Biological warfare agents use living organisms such as bacteria (e.g., *Bacillus anthracis*, the causative agent of anthrax) or viruses (e.g., Variolae, the virus that causes smallpox).

Further, these warfare agents have been subdivided into multiple classes in the public domain. These include choking gases and lung irritants, blister agents (vesicants), blood agents, nerve agents, incapacitants and psychoactive chemicals, harassing or riot-control agents, vomiting agents, herbicides, Napalm, and obscurant smoke and masking agents. The main routes of entry of these substances include inhalation, ingestion, injection, and absorption.

The US Food and Drug Administration (FDA), in collaboration with the US Department of Defense (DOD), continues to support the US military and the nation's counterterrorism efforts. The FDA has helped make critical medical products available for combat readiness so that armed forces are better equipped for combat. It has helped US Special Forces obtain medical products for airborne hospitals used in evacuating battlefield casualties. The FDA has provided consultation and review to help make investigational and licensed medical products such as vaccines and drugs available to combat forces. In developing such warfare agents for counterterrorism, extensive testing for their efficacy and safety is required, especially in animals and humans. A schematic diagram showing the effects of warfare agents and use of the 3Rs concept is shown in Fig. 51.1. The agreement on Mutual Acceptance of Data (MAD; www.oecd.org/chemicalsafety/testing/mutualacceptanceofdata-mad.htm) eliminated the need for duplication of tests in each country before approval or banning of a substance within Organization for Economic Co-operation and Development (OECD) countries, thus reducing animal use.

It is noted that animal testing is highly controversial and has become an emotional issue in recent years (Krishna, 2010; SOT, 2013a,b). Keeping the focus of this chapter in mind, the testing of chemical warfare agents in humans is not practical and is considered unethical, especially at toxic doses. To address this issue, it is important to understand and recognize the growing impact of alternatives to animal and/or human testing. These methods include in vitro toxicity, metabolism, and efficacy/potency testing in a variety of scenarios.

Anadon et al. (2013) highlighted the value of in vitro testing in science. They stated that there is a correlation between toxicological data from in vivo animal studies and in vitro assays. They proposed an integrated in vitro testing strategy by taking into consideration the following: exposure modeling of chemical agents for in vitro testing; data gathering, sharing and read-across for testing a class of chemical; a battery of tests to assemble a broad spectrum of data on different mechanisms of action to predict toxic effects; applicability of the test and the integrated in vitro testing strategies and flexibility to adjust the integrated in vitro testing strategies to test substances (Goldberg and Frazier, 1989; Hartung, 2011; Hartung et al., 2013). However, they emphasize validating in vitro

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FIGURE 51.1 Alternative concept of warfare testing.

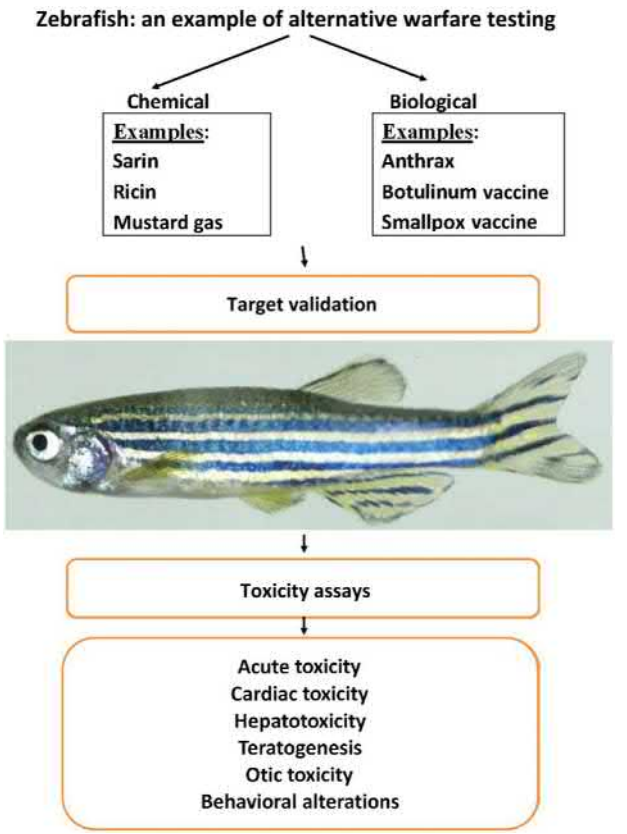

FIGURE 51.2 Zebrafish as an alternative animal model in warfare testing.

systems to ensure reliability and suitability for humans, thus, the data can be invaluable and effective. Schechtman (2002) reviewed the implementation of the 3Rs (refinement, reduction, and replacement) and validation and regulatory acceptance considerations for alternative toxicological test methods and suggested pros and cons of in vitro testing.

Animal testing is an essential part of basic research, especially in inventing and developing effective chemical warfare. Animal testing is defined as the use of nonhumans, such as vertebrate and nonvertebrate animals or organisms, in scientific experimentation in search of answers for the good of mankind and the pursuit of basic knowledge (Krishna et al., 2014). Vertebrate animals ranging from zebrafish to nonhuman primates are commonly used worldwide. However, their use is heavily regulated. An example of a zebrafish testing strategy is shown in Fig. 51.2. More recently, the zebrafish has been identified as an important vertebrate model for studying the development of embryos and pathogenesis of human diseases (Lieschke and Currie, 2007). When compared with mammalian models, experimental results show that zebrafish embryos exhibit similar responses to test agents (Hill et al., 2005). This suggests that the zebrafish model can be used as a bridge between in vitro and in vivo models in the toxicity screening process.

Similarly, invertebrates such as fruit flies (*Drosophila*) and earthworms are also commonly used in research, where applicable, but their use is excluded from the regulations. Around the globe, animal testing is conducted under strict regulatory guidelines which set fundamental standards for the humane use of animals for training, experimentation, biological testing, or other related purposes. These regulations and guidelines require minimizing harm to animals through the 3Rs: reduction, refinement, and replacement, as initially described by Russell and Burch (1959), searching for alternatives including consideration for hierarchical use of species and in vitro methods.

In this chapter, a brief history of chemical warfare use in humans, their classification, use of alternative methods of animal testing, in particular, in vitro toxicology tests, structure–activity relationships, the use of 3Rs including the "Animal Rule" (FDA 2009, 2010) and "human-on-a-chip" to possibly test warfare agents are described.

51.2 Brief history of chemical warfare use

- Poisonous agents have been used as tools of war for thousands of years, even as early as 600 BCE, for example, poisoned arrows, boiling tar, arsenic smoke, and noxious fumes, etc. (www.opcw.org/about-chemical-weapons/history-of-cw-use/).
- In the 1900s, modern chemical warfare began on the battlefields of World War I. Chlorine and phosgene gases were released from canisters on the battlefield and dispersed by wind. The first large-scale attack with chlorine gas occurred on April 22, 1915, at Ypres in Belgium.
- The use of several different types of chemical weapons, including mustard gas (yperite), resulted in 90,000 deaths and over one million casualties during the war. Those injured in chemical warfare suffered from the effects for the rest of their lives; thus the events at Ypres during World War I scarred a generation. By the end of World War I, 124,000 metric tons of chemical agent had been expended. The means of delivery for chemical agents evolved over the first half of the 20th century, increasing these weapons' already frightening capacity to kill and maim through the development of chemical munitions in the form of artillery shells, mortar projectiles, aerial bombs, spray tanks, and landmines.
- After witnessing the effects of such weapons in World War I, it appeared that few countries wanted to be the first to introduce even deadlier chemical weapons onto the World War II battlefields. However, preparations were made by many countries to retaliate in kind should chemical weapons be used in warfare. Chemical weapons were deployed on a large scale in almost all theaters in World Wars I and II, leaving behind a legacy of old and abandoned chemical weapons, which still present a problem for many countries.
- During the Cold War, the United States and the Soviet Union both maintained enormous stockpiles of chemical weapons, amounting to tens of thousands of metric tons. The amount of chemical weapons held by these two countries was enough to destroy much of the human and animal life on Earth.
- Iraq used chemical weapons in Iran during the war in the 1980s, and Iraq also used mustard gas and nerve agents against Kurdish residents of Halabja, in northern Iraq, in 1988.
- The two most recent examples of the use of chemical weapons are the sarin poisoning incident in Matsumoto, a Japanese residential community, in 1994, and the sarin attack on the Tokyo subway in 1995, both perpetrated by the Aum Shinrikyo doomsday cult. These two attacks refocused international attention on the potential use of chemical weapons by terrorists, and on the dangers posed by these chemical weapons.
- The devastating impact chemical weapons have had in the past, and the potential for the use of modern—even more deadly—chemical agents not only by states at war but in other violent conflicts and by nonstate actors, provide the imperative for an international effort to uphold the ban on such weapons and to work toward the complete, global elimination of chemical weapons.

51.3 Top five chemical warfare agents

It has been reported that sarin, ricin, mustard gas, agent 15, and chlorine gas are the top chemical warfare agents (www.livescience.com/39332-5-chemical-warfare-agents.html).

1. *Sarin* is a deadly toxic organophosphate compound with no color, taste, or odor. Though it is produced as a liquid, its low evaporation point allows it to turn into a gas quickly when exposed to the environment. Sarin, also known as GB by military personnel, was originally developed as a pesticide in Germany in 1938, but since then, it has been classified by many national governments as a chemical nerve agent. Nerve agents are the most toxic and fast-acting chemical warfare agents in the world. People exposed to large amounts of sarin quickly lose control over their bodily functions, and, if not treated immediately, can fall into a coma or succumb to respiratory failure.
2. *Ricin* is derived from a common plant, the castor bean (*Ricinus communis*), native to the Mediterranean and Middle East and cultivated elsewhere as an ornamental plant. It is also the source of castor oil, which has many uses in medicine, food, and industry. Ricin is also a highly potent toxin that can kill a person in amounts as small as a few grains of sand.
3. *Mustard gas*, or sulfur mustard ($(Cl\text{-}CH_2CH_2)2S$, is a chemical agent that causes severe burning of the skin, eyes, and respiratory tract. It can be absorbed into the body through inhalation, ingestion, or by contact with the skin or eyes. First used during World War I, the gas is effective at incapacitating its victims en masse. Sulfur mustard is generally colorless in its gaseous state, though it may have a faint yellow or green tint. It is easily recognized by its trademark "mustardy" odor, though some compare its smell to that of garlic, horseradish, or sulfur.
4. *Agent 15* is also called compound 3-quinuclidinyl benzilate, BZ or "Buzz." It is a powerful chemical warfare agent. As one of the most potent psychoactive chemical agents, only a small amount of BZ is needed to produce complete incapacitation. When used as an aerosol, BZ is absorbed through the respiratory system (it has no odor). It can also be absorbed through the

skin or the digestive system. It takes about an hour for BZ to take effect, and the symptoms of exposure include confusion, tremors, stupor, hallucinations, and coma, which can last for more than 2 days. Use of BZ was suspected in the Bosnian conflict in 1995. Similarly, in January 2013, Syrian government troops may have used Agent 15 on rebels.

5. *Chlorine gas* is another chemical agent with a history of use going over 100 years. During World War I, chlorine gas, sometimes known as bertholite, was used by the German army during the Second Battle of Ypres in Belgium. Because chlorine can be pressurized and cooled into a liquid, it can be easily shipped and stored in tanks. When released as a gas, chlorine stays close to the ground and spreads quickly, making it an ideal agent for warfare or terrorism. Though it is used less often today (because more lethal agents exist), chlorine is easy to manufacture and disguise, since it has many other civilian uses such as water sanitation. In 2007, chlorine gas bombs were used to kill dozens of people during the conflict in Iraq.

Toxicology of these warfare agents and many more have been elegantly described elsewhere in this book. Table 51.1 lists a variety of warfare agents. The focus of this chapter is alternatives to animal testing of such warfare agents. The tests mentioned and the strategies described might have to be optimized to suit such a test setting, depending on the type of warfare agent, whether it is liquid, solid, aerosol, or even a biological agent.

TABLE 51.1 Listing of multiple categories of chemical warfare agents.

Blood agents		
Name	**CAS number**	
Arsine	7784-42-1	
Cyanogen chloride	506-77-4	
Hydrogen cyanide	74-90-8	
Vomiting agents		
Name	**CAS number**	**Chemical name**
Adamsite	578-94-9	(10-Chloro-5,10-dihydrophenarsazine)
Diphenylchloroarsine	712-48-1	
Diphenylcyanoarsine	23525-22-6	
Nerve agents		
Name	**CAS number**	**Chemical name**
Cyclohexyl sarin	329-99-7	Cyclohexylmethylphosphonofluoridate
GE	1189-87-3	Phosphonofluoridic acid, ethyl-, 1-methylethyl ester
Sarin	107-44-8	Phosphonofluoridic acid, methyl-, 1-methylethyl ester
Soman	96-64-0	Phosphonofluoridic acid, methyl-, 1,2,2-trimethylpropyl ester
Tabun	77-81-6	Phosphoramidocyanidic acid, dimethyl-, ethyl ester
VE	1189-87-3	Phosphonofluoridic acid, ethyl-, 1-methylethyl ester
Amiton	78-53-5	Phosphorothioic acid, *S*-[2-(diethylamino)ethyl] *O*,*O*-diethyl ester
GV (also GP)	141102-74-1	Phosphoramidofluoridic acid, dimethyl-, 2-(dimethylamino)ethyl ester
VM	21770-86-5	Phosphonothioic acid, methyl-, *S*-[2-(diethylamino)ethyl] *O*-ethyl ester
VX	50782-69-9	Phosphonothioic acid, methyl-, *S*-[2-[bis(1-methylethyl)amino]ethyl] *O*-ethyl ester
Blister agents		
Name	**CAS number**	**Chemical name**
Mustard gas	505-60-2	Bis(2-chloroethyl) sulfide
Nitrogen mustard	0538-07-08	Bis(2-chloroethyl)ethylamine

(*Continued*)

TABLE 51.1 (Continued)

Blister agents

Name	CAS number	Chemical name
Nitrogen mustard	51-75-2	Bis(2-chloroethyl)methylamine
Nitrogen mustard	555-77-1	Tris(2-chloroethyl)amine
Phosgene oxime	1794-86-1	
2-Chloroethyl ethyl sulfide	0693-07-02	
Sesqui mustard	3563-36-8	1,2-Bis(2-chloroethylthio)ethane
Ethyldichloroarsine	598-14-1	
Lewisite	541-25-3	2-Chlorovinyldichloroarsine
Lewisite-2	40334-69-8	Bis(2-chlorovinyl)chloroarsine
Lewisite-3	40334-70-1	Arsine, tris(2-chloroethenyl)-
Methyldichloroarsine	593-89-5	Arsonous dichloride, methyl-
Mustard/lewisite		
Phenyldichloroarsine	696-28-6	Arsonous dichloride, phenyl-

Riot control/tear agents

Name	CAS number	Chemical name
Bromobenzylcyanide	16532-79-9	4-Bromophenylacetonitrile
Chloroacetophenone	532-27-4	
Chloropicrin	1976-06-02	
CNB		10% CN, 45% benzene, 45% carbon tetrachloride
Fentanyl	437-38-7	Propanamide, *N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl]-
CNC		30% CN in chloroform
CNS		23% CN, 38% chloropicrin, 38.4% chloroform
Dibenz-(b,f)-1,4-oxazepine	0257-07-08	
O-Chlorobenzylidenemalononitrile	2698-41-1	CS1, CS2, CSX all have CS as agent

Pulmonary (choking) agents

Name	CAS number	Chemical name
Ammonia	7664-41-7	
Chlorine	7782-50-5	
Hydrogen chloride	7647-01-0	
Phosgene	75-44-5	
Diphosgene	503-38-8	Trichloroacetyl chloride
Titanium tetrachloride	7550-45-0	
Nitric oxide	10102-43-9	
Perflurorisobutylene	382-21-8	1,1,3,3,3-Pentafluoro-2-(trifluoromethyl)-1-propene
Red phosphorous	7723-14-0	
Sulfur trioxide-chlorosulfonic acid		(Smoke mixture)
Sulfur trioxide	7446-11-9	
Chlorosulfonic acid	7790-94-5	
Zinc oxide	1314-13-2	

(*Continued*)

TABLE 51.1 (Continued)

Pulmonary (choking) agents		
Name	**CAS number**	**Chemical name**
Agent 15 (similar to BZ)		
3-Quinuclidinyl benzilate	6581-06-2	3-Quinuclidinyl benzilate
Delta-9-THC	33086-25-8	Delta-9-tetrahydrocannabinol
China White	79704-88-4	alpha-Methylfentanyl
Fentanyl	437-38-7	Propanamide, *N*-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl]-
LSD	50-37-3	D-Lysergic acid *N*,*N*-diethylamide
Phenothiazine	92-84-2	Phenothiazine
Thorazine	50-53-3	Chlorpromazine

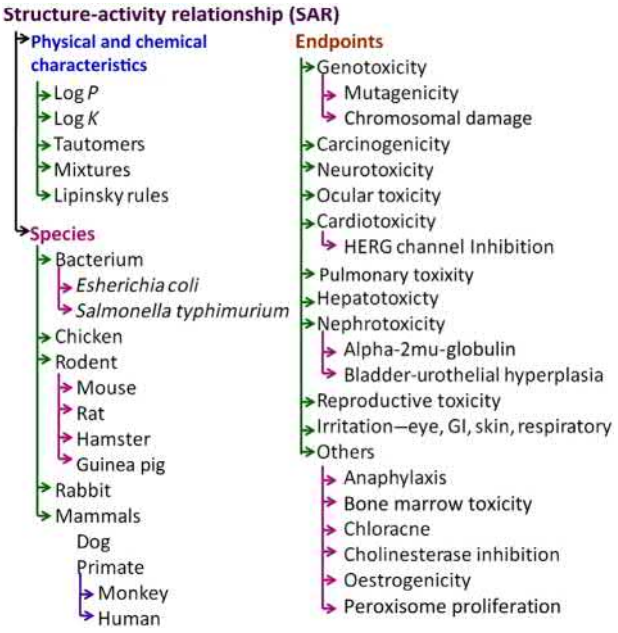

FIGURE 51.3 In silico endpoints commonly used to achieve 3Rs.

51.4 The concept of 3Rs

The concept 3Rs is further explained by the National Center for the Replacement, Reduction and Refinement of animals in research (NC3R, 2013; http://eslav-eclam.org/other-associations/nc3rs). These are a widely accepted ethical framework for conducting scientific experiments using animals humanely. These include:

- *Replacement*: Use methods that avoid or replace the use of animals defined as "protected" under the Animals (Scientific Procedures) Act 1986, amended 2012 (ASPA) in an area where they would otherwise have been used. "Protected" animals are all living vertebrates (other than man), including some immature forms, and cephalopods (e.g., octopus, squid, cuttlefish). This can be:
  - Absolute replacements—those which do not involve animals at any point;
  - Relative replacements—those which avoid or replace the use of "protected" animals.
  - Examples include:
    - Computer modeling, such as Lhasa (2014) (Fig. 51.3);
    - Human volunteers, for example, for noninvasive imaging studies;
    - Invertebrates, such as *Drosophila* (fruit fly) and nematode worms;
    - Immature forms of vertebrates: mammal, bird, and reptile embryos, up to the last third of their gestation or incubation period, larval forms of amphibians and fish, until the stage where they become capable of independent feeding, cephalopods until the point at which they hatch;
    - In vitro methodologies, utilizing established human or animal cell lines, animal cells, tissues, and organs from animals killed by a humane method, abattoir material from the meat industry (Figs. 51.4 and 51.5).
- *Reduction*: Use methods which minimize animal use and enable researchers to obtain comparable levels of information from fewer animals or to obtain more information from the same number of animals, thereby further reducing future use of animals.
  - Examples include:
    - Sharing data and resources, such as the MAD agreement;
    - Improved experimental design and statistical analysis;

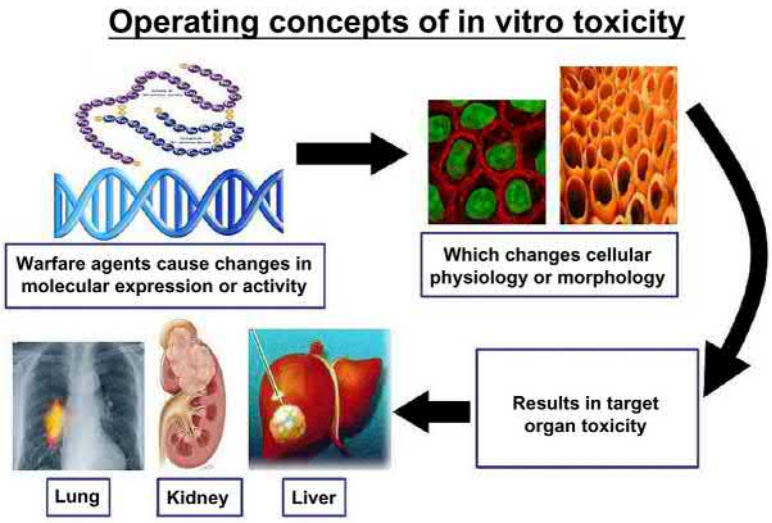

FIGURE 51.4 Warfare agents cause changes at the molecular level (DNA or protein), which are expressed at the cellular level, which in turn result in target organ toxicity (e.g., liver, kidney), which can be studied and extrapolated to whole animal or humans based on in vitro studies.

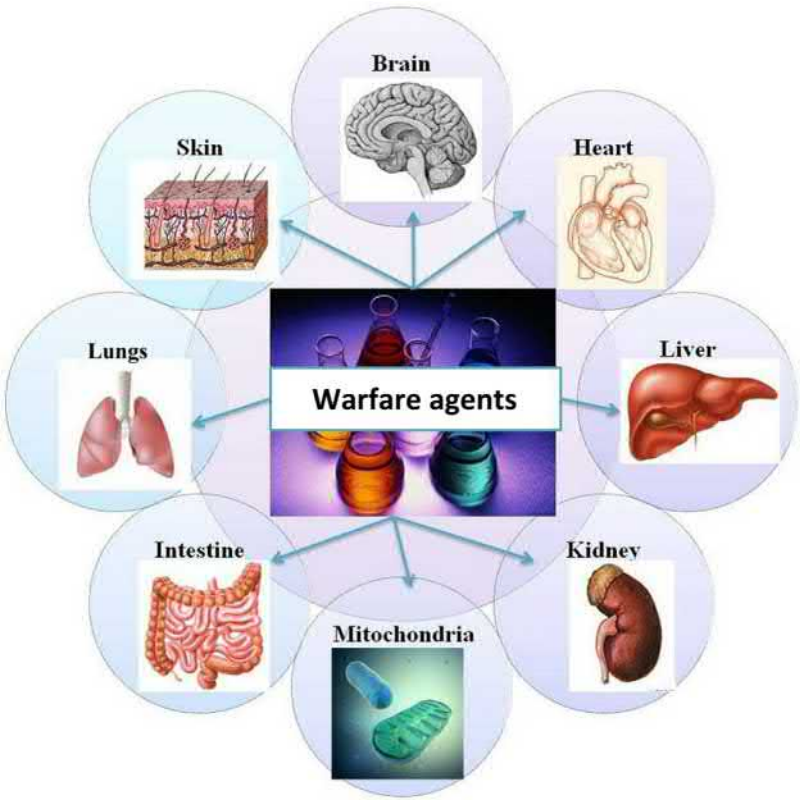

FIGURE 51.5 Warfare agents interact with various cells, organelles, and organ systems to produce their toxicological effects. These effects can now be studied using various in silico or in vitro tools minimizing the use of animal testing.

– Integrated genotoxicity testing using fewer animals and collecting most information. The concept of integrating toxicity assessment, where with fewer animals a variety of relevant toxicity data can be collected and evaluated (Krishna et al., 1994, 1995a,b, 1998, 2000; Fig. 51.6). As an example, an integrated in vivo genotoxicity testing philosophy and a practical approach, as applied to pharmaceuticals, is currently practiced and recommended by the International Conference on Harmonization (ICH) guidance (ICH, 2006). In this case, a rodent (primarily rat) micronucleus assay is integrated with routine 2- to 4-week repeat dose toxicity and toxicokinetic studies. This approach has several advantages: (1) it utilizes the general principles of toxicology that govern the overall toxicity

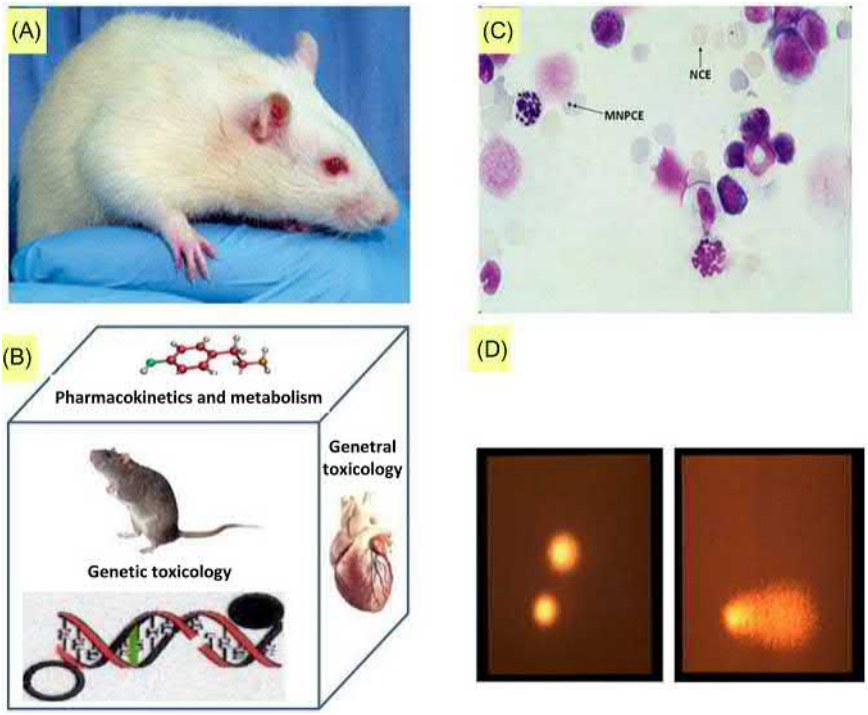

FIGURE 51.6 Integrated in vivo genotoxicity of warfare agents can be evaluated using samples obtained during the general toxicity study: (A) samples are obtained from the animal (e.g., rat), (B) the test agent is exposed to in vivo biochemical processes, including metabolism and opportunity to interact with the DNA, thereby providing an intact animal with homeostatic assessment of the genotoxic potential of test agent, and (C) the blood sample/bone marrow is obtained and stained to detect for the presence of micronuclei in polychromatic erythrocytes (PCE); if positive, then the test agent is considered as clastogenic. (D) Cells, particularly from the liver, are processed and tested for DNA strand breaks in the COMET assay. The breaks in the DNA are observed as a comet-shaped migration of DNA from the nucleus of the cell following gel electrophoresis in treated compared to intact round nucleus suggesting unaffected DNA (control).

profile of a test substance; (2) factors such as the dose and/or route of test agent administration, metabolism, principles of toxicokinetics, and saturation of defense mechanisms are considered in evaluating genotoxicity; (3) it uses the concept of administering multiple tolerable doses aiding in achieving steady-state plasma test agent levels, which is more relevant for risk assessment compared to high acute doses; and (4) it helps minimize the amount of test agent, number of animals used, and other resources. This integration approach can be extended to other toxicology studies and other relevant genotoxicity endpoints may be assessed. Based on experience reported in the literature, integrating micronucleus assessment in routine toxicology testing is promising and should be utilized when practical. A number of genotoxicity endpoints can be easily evaluated in peripheral blood lymphocytes of exposed subjects by genetic monitoring.

- Modern imaging techniques
  - Noninvasive, whole-body imaging of small animals using techniques such as X-ray, CT, SPECT, PET, and MRI, is helping to reduce the number of animals used in basic research and testing of chemical warfare agents. Fig. 51.7 illustrates such an example. The same animal can be imaged multiple times in order to monitor visually, often in real time, the progression or regression of infection or disease. This avoids the need to sequentially sacrifice animals at different time points, allowing significant reductions in the number of animals used per study.
- *Refinement*: Use improvements to scientific procedures and husbandry which minimizes actual or potential pain, suffering, distress, or lasting harm and/or improves animal welfare in situations where the use of animals is unavoidable. Refinement applies to the lifetime experience of the animal. There is evidence that refinement not only benefits animals, but can also improve the quality of research findings. Fig. 51.8 shows a unique refinement of housing of mice.
  - Examples include:
    - Noninvasive techniques;
    - Appropriate anesthetic and analgesic regimens for pain relief;
    - Training animals to voluntarily cooperate with procedures (e.g., blood sampling) so that they have greater control over the procedure and are less stressed;
    - Accommodation and environmental enrichment which meets the animals' physical and behavioral needs (e.g., providing opportunities for nesting for rodents).

51.5 International cooperation on alternative test methods

In 2009, the United States, Canada, Japan, and the EU signed a memorandum of cooperation that could reduce

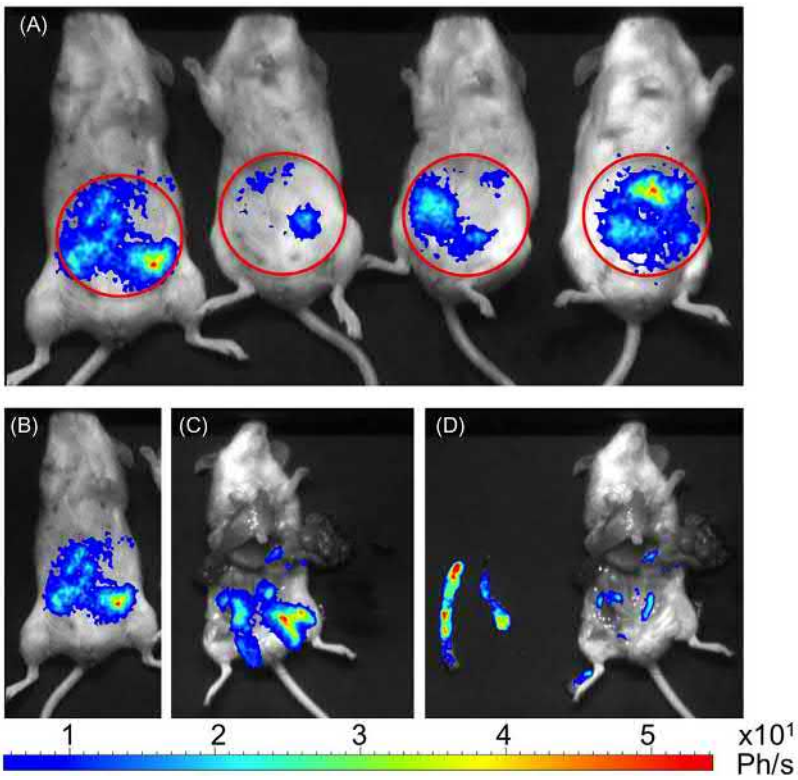

FIGURE 51.7 Localization of bioluminescent parasites in intraabdominal mesenteric fat in infected mice. Infection was monitored by bioluminescence imaging (BI) technique. On day 40 postinfection (A), mice showed intraperitoneal parasites localized by BI. One representative mouse (B) was dissected and the adipose tissue localization was verified in situ by BI (C) and confirmed after removing the adipose tissue and reimaging (D).

FIGURE 51.8 The "mouse house" is a refinement using a transparent, red, plastic house, which enables the mice to perform natural behaviors such as nesting, hiding, and climbing, which is important for their welfare. The house appears dark to the mice, yet the transparent walls allows the scientist to make observations without disturbance (www.scanbur.com).

the number of animals required for consumer product safety testing worldwide (International Cooperation on Alternative Test Methods [ICATM]; http://ntp.niehs.nih.gov/?objectid = 62A650A4-DD4B-D0A8-C26C7AE0A57F82E8). A similar approach can be used for evaluating chemical warfare agents. The agreement is hoped to yield globally coordinated scientific recommendations on alternative toxicity testing methods that should speed their adoption in each of these countries, thus reducing the number of animals needed for safety testing. A flowchart of this is shown in Fig. 51.9.

ICH: The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use;

OECD: The Organization for Economic Cooperation and Development has a Test Guideline program that deals with chemicals;

ICCR: The International Cooperation on Cosmetics Regulation (ICCR) is an international group of cosmetic regulatory authorities from the United States (FDA), Japan (Ministry of Health, Labor, and Welfare), the EU (EC, DG Enterprise), and Canada (Health Canada). This multilateral framework maintains the highest level of global consumer protection, while minimizing barriers to international trade.

The alternative toxicology methods listed and suggested may be used for chemical warfare agents, as applicable. Nonanimal methods for toxicity testing currently being used are listed in Table 51.2. This was initially compiled by an organization called AltTox (2013), but was modified to serve the purposes of this chapter. These methods are also used by organizations such as Registration, Evaluation, Authorization and Restriction of Chemicals (REACH, 2013).

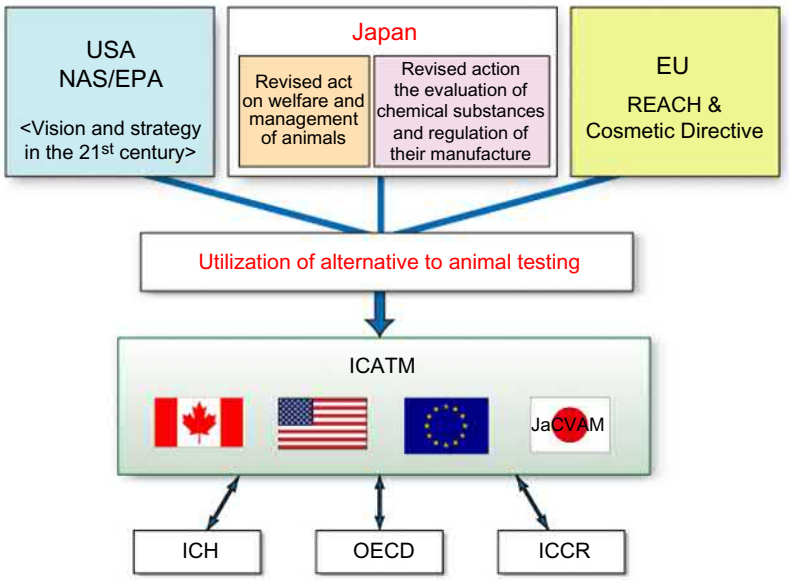

FIGURE 51.9 International Cooperation on Alternative Test Methods (ICATM).

TABLE 51.2 Alternative test methods and testing strategies for chemical warfare agents.

Endpoint	Method name	Test type
Acute mammalian toxicity (oral)	Acute toxic class method	In vivo
	Fixed dose procedure	In vivo
	Up-and-down procedure	In vivo
	Normal human keratinocyte neutral red uptake (NHK NRU) assay	In vitro
	Balb/c 3T3 neutral red uptake assay	In vitro
Acute mammalian toxicity (hematotoxicity)	Colony forming unit-granulocyte/macrophage assay for acute neutropenia in humans	In vitro
Acute mammalian toxicity (inhalation)	Acute toxic class method	In vivo
Acute toxicity testing of pesticides	Guidance for waiving or bridging of mammalian acute toxicity tests for pesticides (acute oral, dermal, inhalation; primary eye and dermal; dermal sensitization)	In vivo
Biologics and vaccines	Enzyme-linked immunosorbent assay (ELISA) for swine erysipelas vaccines batch potency testing	In vitro
	ELISA for human tetanus vaccines batch potency testing	In vitro
	Toxin binding inhibition test for human tetanus vaccines batch potency testing	In vitro
	Batch potency testing of erythropoietin concentrated solution	In vivo
	Deletion of target-animal safety test for batch safety testing of veterinary vaccines after consistency in 10 consecutive batches	NA
	ELISA for in vitro batch potency testing of *Leptospira* veterinary vaccines	In vitro
	Use of humane endpoints in animal testing of veterinary biologics, including rabies vaccines	In vivo
	Veterinary vaccine potency assays: exemptions from standard requirements tests; master reference qualification and requalification	NA
	Cell-based assay for stability and potency of botulinum neurotoxin type A products	In vitro
	Alternative test procedure for tuberculin, purified protein derivative (PPD) bovis, intradermic	In vivo
Carcinogenicity	Three cell transformation assays (CTA): Syrian hamster embryo (SHE) CTA performed at pH 6.7, SHE CTA performed at pH 7.0, and BALB/c 3T3 CTA	In vitro

(*Continued*)

TABLE 51.2 (Continued)

Endpoint	Method name	Test type
Chronic toxicity	Ending 1-year dog studies of pesticides	In vivo
Dermal absorption/penetration	In vitro skin absorption methods	In vitro
Ecotoxicity	Acute aquatic toxicity: upper threshold concentration step-down approach	In vivo
	Acute avian toxicity (oral): sequential testing procedure to minimize numbers of birds used	In vivo
	Fish embryo toxicity	In vivo
Endocrine active substances	Androgen receptor-binding assay (rat prostate cytosol)	Ex vivo
	Aromatase inhibition assay (human recombinant)	In vitro
	Stably transfected trans-activation in vitro assays to detect estrogen receptor agonists	In vitro
	Estrogen receptor-binding assay rat uterine cytosol (ER-RUC)	Ex vivo
	H295R steroidogenesis assay	In vitro
	US EPA tier 1 screening battery	In vitro/ in vivo
	BG1Luc ER TA test method for estrogen agonists and antagonists	In vitro
Eye corrosion	Bovine corneal opacity permeability test	Ex vivo
	Cytosensor microphysiometer modified	In vitro
	Fluorescein leakage	In vitro
	Hen's egg test-chorioallantoic membrane	In vitro
	Isolated chicken eye test	Ex vivo
	Isolated rabbit eye test	Ex vivo
	Routine use of topical anesthetics, systemic analgesics, and humane endpoints	In vivo
	Sequential testing strategy for eye irritation and corrosion	In vitro/ ex vivo/ in vivo
Eye irritation	Cytosensor microphysiometer modified	In vitro
	Rabbit low-volume eye test	In vivo
	Routine use of topical anesthetics, systemic analgesics, and humane endpoints	In vivo
	Sequential testing strategy for eye irritation and corrosion	In vitro/ ex vivo/ in vivo
Genotoxicity	Bacterial reverse mutation (Ames) test	In vitro
	In vitro cell gene mutation test	In vitro
	In vitro chromosomal aberration test	In vitro
	In vitro mammalian cell micronucleus test	In vitro
	In vitro sister chromatid exchange test	In vitro
	In vitro unscheduled DNA synthesis test	In vitro
	Saccharomyces cerevisiae gene mutation assay	In vitro
	S. cerevisiae mitotic recombination assay	In vitro
Immunotoxicity/skin sensitization	Local lymph node assay (LLNA)	In vivo
	Reduced LLNA: rLLNA	In vivo
	Nonradiolabeled LLNA: DA	In vivo
	Nonradiolabeled LLNA: BrdU-ELISA	In vivo
	LLNA for potency categorization of skin sensitizers	In vivo

(*Continued*)

TABLE 51.2 (Continued)

Endpoint	Method name	Test type
Phototoxicity	3T3 neutral red uptake phototoxicity test	In vitro
	3T3 NRU phototoxicity test: application to UV filter chemicals	In vitro
Preclinical and nonclinical safety studies for drug development	Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3(R2) (harmonized guidance can reduce use of animals)	In vivo
	Preclinical safety evaluation of biotechnology-derived pharmaceuticals, ICH S6 (R1)	In vivo
Pyrogenicity	Human whole blood IL-1	In vitro
	Human whole blood IL-6	In vitro
	Human cryopreserved whole blood IL-1	In vitro
	PBMC IL-6	In vitro
	MM6 IL-6	In vitro
	Limulus amebocyte lysate (LAL) test	In vivo/ in vitro
Reproductive and developmental toxicity	Embryonic stem cell test for embryotoxicity	In vitro
	Micromass embryotoxicity assay	Ex vivo
	Whole rat embryotoxicity assay	Ex vivo
	Extended one-generation reproductive toxicity study	In vivo
Skin corrosion	EST-1000 human reconstructed epidermis	In vitro
	Membrane barrier corrosivity test method (Corrositex)	In vitro
	EpiSkin human skin model	In vitro
	EpiDerm human skin model	In vitro
	Rat skin transcutaneous electrical resistance assay	Ex vivo
	SkinEthic human skin model	In vitro
	Vitrolife-Skin human reconstructed epidermis	In vitro
Skin irritation	EpiSkin skin irritation test (with MTT reduction)	In vitro
	EpiDerm skin irritation test (with MTT reduction)	In vitro
	EpiDerm SIT model (EPI-200)	In vitro
	SkinEthic RHE model	In vitro

51.6 Alternatives to animal testing of chemical warfare agents

Greenfield et al. (2014) report that the US Army Medical Research Institute of Chemical Defense (USAMRICD) has been commissioned with a mission to develop medical countermeasures against exposure to chemical warfare agents as well as against agents of biological origin. Over the years, this organization has reduced the number of animals used in its research protocols by 92%. Based on a report by the US House of Representatives Committee on the Armed Services regarding the use of animals in Department of Defense (DOD) military experiments, initiatives to promote alternatives to reduce, refine, and replace (3Rs) were undertaken in 1992. During this time, while adopting the 3Rs, the DOD also added a fourth R, responsibility. This organization developed a unique concept called management by objective program based on science and technology objectives. One such major objective was to develop reduction, refinement, and

replacement strategies for the use of animals in research. The goal was to develop technologies that would incrementally reduce reliance on animal and human subject research and improve the experimental conditions using animals. This further requires introducing a minimum of one improvement per year in experimental protocols using animals.

The scientists at the USAMRICD have taken advantage of emerging computer and biological technologies in the design of molecular modeling software and in the development and maintenance of cell culture models to meet the animal reduction goals. In addition, scientists, where possible, have adopted the use of less sentient animal species.

As an example, computer modelings of the molecular structure of nerve agents, physiological enzymes, and neurotransmitters are used to predict and eventually determine how these chemical compounds interact at the molecular level. A test compound that inhibits acetylcholinesterase (AChE) aging has also been studied via computer modeling to determine the specific molecular events that occur at the peripheral anionic site of AChE, which subsequently prevents a potent AChE inhibitor, such as the nerve agent sarin, from irreversibly binding to the AChE molecule (Khan et al., 2000). With this knowledge the most likely candidates for chemical intervention in the prevention or treatment of nerve agent exposure can be determined. To this end, multiple cell lines or cell cultures are used in research and have led to a reduction of animal usage in research programs on blistering chemical warfare agents. In efforts to evaluate the effects of blister agents, or vesicants, particularly sulfur mustard, and to develop medical countermeasures against such agents, alternatives to animals have proven particularly useful. Several cell line or culture systems, such as peripheral blood lymphocytes, human epidermal keratinocytes, and the HeLa, a human epithelial tumor line, have been adapted and developed by scientists into valuable models for studying vesicant injury. In addition, studies have used a commercial human skin equivalent model and skin biopsies from the Cooperative Human Tissue Network. The USAMRICD also has developed the technology to process and generate a human epidermal model, which possesses typical structural components of human epidermis in vivo to include hemidesmosomes, anchoring filaments, and elements of a true basement membrane. The use of these alternative models has led especially to a decrease in the number of rodents required for these studies.

The USAMRICD also promotes the use of less sentient or less regulated animal species, for example, the use of *Aplysia californica*, a large naked marine mollusk or gastropod with anterior sensory tentacles, commonly referred to as the sea hare or sea slug. This slug possesses large and discrete neural ganglia and its unique neural anatomy that makes these invertebrate animals an excellent and widely used model for neural transmission and neurotoxin research. Murphy and Glanzman (1997) have described "classical conditioning" based on neurological studies in *Aplysia*. It appears to be mediated, in part, by long-term potentiation due to activation of *N*-methyl-D-aspartate-related receptors. In certain sulfur mustard experimental protocols the USAMRICD has used SKH-1 hairless mice as an appropriate substitute for the hairless guinea pig. The main histological feature of skin lesions produced in a SKH-1 hairless mouse following exposure to sulfur mustard is the formation of microblisters at the dermal−epidermal junction. Similar microblisters also occur in sulfur mustard lesions of hairless guinea pigs. Whereas laboratory mice are not subject to the Animal Welfare Act, provisions for their care and use are contained in the Guide for the Care and Use of Laboratory Animals.

51.7 Animal efficacy rule

The animal efficacy pathway was formed shortly after the terror attacks of 2001, which included a harrowing series of incidents in which spores of anthrax were sent through the US Postal Service, killing several people and sickening many more (www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart = 314&showFR = 1&subpartNode = 21:5.0.1.1.4.9).

In response to the attacks, legislators and regulators determined that a regulatory process needed to be established to test the safety and effectiveness of so-called medical countermeasures, while taking into account the extremely dangerous nature of the pathogens against which they would need to protect their subjects. The problem, at its most basic level, was testing a drug product in humans which could potentially kill them, the answer to that question was, rather simply, we do not test in humans for efficacy. Instead, the FDA ultimately established the animal efficacy rule, which is an approval pathway through which manufacturers can apply for conditional approval of a medical countermeasure based on efficacy testing in human analogs, such as pigs or chimpanzees, or other appropriately relevant models. Safety testing is still conducted in humans.

Pyridostigmine bromide is the first drug approved under the "animal efficacy rule" that allows use of animal data for evidence of the drug's effectiveness for certain conditions when the drug cannot be ethically or feasibly tested in humans.

The "animal efficacy rule," which became effective on June 30, 2002, is an important component of the FDA's efforts to make medical countermeasures available to treat or prevent the effects of biological and chemical agents. The "animal efficacy rule" enabled the FDA to approve

pyridostigmine bromide to increase survival following soman poisoning despite the impossibility of ethically conducting human studies on the effectiveness of the drug.

The nerve agent soman causes loss of muscle control and death from respiratory failure. Evidence of the effectiveness of pyridostigmine bromide as a pretreatment for exposure to soman was obtained primarily from studies in monkeys and guinea pigs. This evidence shows that administration of the drug before exposure to soman, together with atropine and pralidoxime given after exposure, increases survival. The FDA believes that, based on the animal evidence of effectiveness, pyridostigmine bromide is likely to benefit humans exposed to soman.

The agency's safety assessment is based on long-term use of pyridostigmine bromide, first approved by the FDA in 1955, to treat a neuromuscular disease called myasthenia gravis. The Department of the Army has submitted data from multiple controlled trials and uncontrolled clinical experience demonstrating pyridostigmine bromide is well-tolerated at the doses intended for military use. The dose used for myasthenia gravis is higher than the dose used for pretreatment to protect against soman.

To use this potentially lifesaving drug correctly, military personnel must carefully follow instructions and use the drug only under specific circumstances. For example, if US troops faced the threat of exposure to soman, they would be given instructions to take pyridostigmine bromide every 8 h prior to the anticipated exposure. Soldiers would be warned that the drug is not effective and should not be taken at the time of, or following, exposure to soman.

The troops are to use the drug in conjunction with other protective measures, including chemical protective masks and battle dress garments. Furthermore, effectiveness depends on the rapid use of the antidotes atropine and pralidoxime and discontinuation of pyridostigmine bromide at the first indication of nerve gas exposure. The Department of Defense plans to provide all military personnel with extensive training, prior to deployment, on the proper use of pyridostigmine bromide, as well as other methods used in the prevention and treatment of nerve agent poisoning.

A leaflet that explains the drug's use, benefits, and side effects will be provided to military personnel when the drug is distributed. The leaflet advises that pyridostigmine bromide should not be used by persons who have a history of bowel or bladder obstruction, or sensitivity to certain medicines used during surgery (like physostigmine). Side effects that may occur include stomach cramps, diarrhea, nausea, frequent urination, headaches, dizziness, shortness of breath, worsening of peptic ulcer, blurred vision, and watery eyes.

The approved dose of pyridostigmine bromide for soman pretreatment is one 30-mg tablet every 8 h. The leaflet states that pyridostigmine should be started at least several hours before exposure to soman and emphasizes that it must be discontinued upon exposure to nerve gas, at which point the antidotes atropine and pralidoxime are given.

During the Gulf War, the FDA had allowed distribution of pyridostigmine bromide under its Investigational New Drug provisions because pretreatment with this drug had the potential to help save lives if nerve agents were used.

Johnson & Johnson's Levaquin (levofloxacin), an antibiotic intended to treat pneumonic plague, became the first product to receive FDA (2012a) approval via the Animal Rule. The product received approval based on testing in African green monkeys infected with pneumonic plague. None of the placebo control group survived contact with the virus, while 94% of the Levaquin group survived.

The FDA's (2012b) approval of raxibacumab marks just the second approval under the Animal Rule, but also the first monoclonal antibody. The drug is also notable in that it is intended to treat inhalation anthrax—the pathogen perhaps most responsible for the formation of the animal efficacy rule.

Raxibacumab was tested in one study involving monkeys and three involving rabbits. As with Levaquin, none of the animals in the control groups survived being infected with inhalation anthrax, while 64% of monkeys and 44% of rabbits in one study treated with raxibacumab survived. The FDA also said GSK's studies showed the drug was an improvement over existing antibiotic therapies, according to the animal studies. It is said that the drug is specifically aimed at defending against another bioterrorism-type event. Although antibiotics are approved to prevent and treat anthrax infection, raxibacumab is the first approved agent that acts by neutralizing the toxins produced by *B. anthracis*.

- *Improvements to the Animal Rule*

  Both approvals come as FDA and other government entities are looking into ways to refine the Animal Rule process and the protections it affords to the human subjects it is intended to protect.

  Members of the Alliance for Biosecurity (Gronvall et al., 2007) have made three recommendations for effective implementation of the Animal Rule.
  - First, the Department of Health and Human Services (HHS), the FDA and other US agencies provide strategic direction about how countermeasures may be used. This will aid development and testing.
  - Second, the FDA, along with the National Institute of Allergy and Infectious Diseases (NIAID), should actively develop scientific consensus on animal models for specific disease threats.
  - Third, the FDA should develop a consistent interpretation of the Rule within the agency.

- Aebersold (2012) reviewed the FDA experience with medical countermeasures under the Animal Rule. The author concludes that even though only a few drugs or biologicals have been approved since the Animal Rule became effective, several investigational drugs have been placed in the National Strategic Stockpile for use as medical countermeasures, if needed.
- The FDA has also clarified suggested issues, one of which is an animal model qualification program. The qualification process is limited to animal models used for product approval under the Animal Rule (www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284078.htm). A qualified model may be used for efficacy testing in development programs for multiple investigational drugs for the same targeted disease or condition. Such animal models are considered to be product-independent (i.e., not linked to a specific drug).

The Animal Rule states that the FDA can rely on the evidence from animal studies to provide substantial evidence of the effectiveness of a drug only when all of the following four criteria, quoted below, are met:

a. There is a reasonably well-understood pathophysiological mechanism of the toxicity of the substance and its prevention or substantial reduction by the product;
b. The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting the response in humans;
c. The animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity; and
d. The data or information on the kinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allows selection of an effective dose in humans.

The FDA (2003) approved pyridostigmine bromide for combat use by US military personnel to protect them from the lethal effects of the nerve gas soman (www.fda.gov/Drugs/EmergencyPreparedness/BioterrorismandDrugPreparedness/ucm130342.htm).

Sullivan et al. (2009) have reviewed the usefulness of the Animal Rule in the development and regulatory approval process of Ebola virus. Ebola virus infection is a highly lethal disease for which there are no effective therapeutic or preventive treatments. Several vaccines have provided immune protection in laboratory animals, but because outbreaks occur unpredictably and sporadically, vaccine efficacy cannot be proven in human trials, which is required for traditional regulatory approval. The FDA has introduced the "Animal Rule," to allow laboratory animal data to be used to show efficacy when human trials are not logistically feasible. In this review, the authors describe immune correlates of vaccine protection against Ebola virus in animals. This research provides a basis for bridging the gap from basic research to human vaccine responses in support of the licensing of vaccines through the Animal Rule. Table 51.3 lists a few agents approved using the Animal Rule.

51.8 Human-on-a-chip

Hartung and Zurlo (2012) have discussed various alternative approaches for medical countermeasures to biological and chemical terrorism and warfare. One such approach focuses on the development of a human-on-a-chip, as shown in Fig. 51.10. This involves the combination of different three-dimensional (stem) cell-based organ equivalents combined with microfluidics. The prospects of such approaches, their impact on the field of alternative approaches, and necessary complementary activities are discussed. They emphasize the need to adapt quality assurance measures and experiences from validation while executing such newer approaches.

Similarly, scientists at the Edgewood Chemical Biological Center (ECBC) and academic collaborators are performing research on organoids (small swatches of human tissue) on microchips (www.kurzweilai.net/human-on-a-chip). This research focuses on in vitro human organ constructs such as for the heart, liver, lung, and circulatory system, in communication with each

TABLE 51.3 Agents approved using the "Animal Rule."

	Compound	Company	Purpose
1	Abthrax (Raxibacumab)	Glaxo Smith-Kline (GSK)	Anthrax vaccine
2	Cynokit	Orphan Medical Inc/Merck Sante	Cyanide Poisoning
3	Ciprofloxacin	Bayer	Anthrax
4	Levofloxacin	Janssen Pharmaceuticals	Plague (*Yersinia pestis*)
5	Pyridostigmine bromide	US Army	Soman

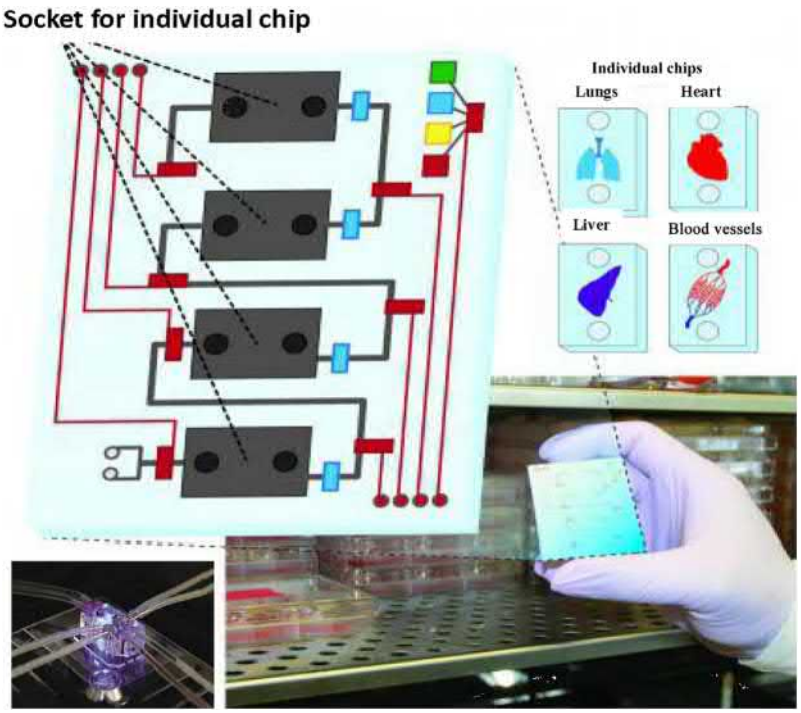

FIGURE 51.10 "Human-on-a-chip" in vitro human organ constructs (for heart, liver, lung, and the circulatory system) in communication with each other used to assess the effectiveness and toxicity of warfare agents that are relevant to humans.

other. The goal is to assess the effectiveness and toxicity of drugs in a way that is relevant to humans and their ability to process these drugs. It is said that "the screening models will be used to assess the efficacy and safety of medical mitigation procedures and countermeasures for the soldier and the nation as a whole."

Each organ-on-a-chip is about the size of a thumb drive and is an "organoid" (a structure that resembles an organ in appearance or function), designed to mimic the properties of an actual human organ. The organoids are created by induced pluripotent stem cells made from adult skin cells. They comprise multiple layers of cells growing on a membrane, connected to each other by microfluidics (tiny microchannels) that copy the function of blood vessels. Their primary purpose is to take the place of animal research. It is proposed that animal tests do not always give the results seen in people. Due to the species-specific differences by which compounds are metabolized, a drug tested on a laboratory rat does not always translate well to a human. In some cases, such as with asthma, no animal testing can mimic the human response. Since human-on-a-chip is made from human cells, it is the next best thing. Human tissue reacts like human tissue.

51.8.1 New predictive models of toxicity

Researchers anticipate that new predictive models of toxicity will result from the more accurate human-on-a-chip testing, saving time and money. This technology is hoped to result in fewer test failures than animal studies. The ECBC houses the only laboratories in the United States that the Chemical Weapons Convention permits to produce chemical warfare agents for testing purposes. It is thought that the ECBC will test the human-on-a-chip against chemical warfare agents to learn more about how the body will respond to agent exposure and explore various treatment options for exposures. For the military, human-on-a-chip research is hoped to save lives.

Labant (2014) points out using in vitro ADME-Tox assays results in more biological data from each experiment. The use of specialized instrumentation such as a single-stage accelerator mass spectrometer (SSAMS) is suggested so trace amounts of metabolites that can be identified and quantitated (Fig. 51.11). Similarly, use of flow cytometry in toxicological testing has truly enhanced the collection of large amounts of data from each experimental units either in vivo or in vitro while reducing animal use (Krishna et al., 1993; Criswell et al., 1998a,b, 2003; Darzynkiewicz et al., 2011; Dertinger et al., 2011; Willjam et al., 1991).

Examples of alternative studies conducted using a variety of warfare agents are briefly listed below. Worek et al. (2007) described the use of highly toxic organophosphorus (OP) warfare nerve agents and underline the necessity for effective medical treatment. Acute OP toxicity is primarily caused by the inhibition of AChE. Reactivators (oximes) of inhibited AChE are a mainstay of treatment. In vitro studies with human tissue have enabled the evaluation of oxime efficacy without animal

FIGURE 51.11 The single-stage accelerator mass spectrometer is a robust and ultrasensitive bioanalytical tool. AMS technology can provide a compound and matrix-independent platform that enables innovative in vitro and in vivo study designs.

experiments and with no need for interspecies extrapolation. Dorandeu et al. (2007) emphasize the use of swine, especially minipigs, based on similarities in a number of interesting biological and physiological characteristics (skin, cardiovascular), as an alternative to other commonly used species such as dogs, macaques, and marmosets. Szinicz et al. (2007) described the development of antidotes against chemical warfare agents as "orphan drugs" given that these poisonings are rare. Computer models are being established to estimate the therapeutic effect of an antidote in various human poisoning scenarios. This approach compensates for the lack of human clinical pharmacodynamic studies given the obvious ethical issues preventing human volunteer studies with these agents. Krishnan et al. (2009) and Reddy et al. (2011a,b) tested a variety of warfare agents in multiple alternative methods including short-term genotoxicity and physiologically based pharmacokinetics modeling of warfare agents, for example, cyclotrimethylenetrinitramine, ethylenediaminedinitrate, diethylenetriaminetrinitrate, and 3-nitro-1,2,4-triazol-5-one.

51.9 Concluding remarks and future directions

Alternatives in animal testing have come a long way in thought and action. Over the years, scientists have continuously improved such methods so that the goals of 3Rs are achieved. For example, the roadmap for the development of alternative (nonanimal) methods for systemic toxicity testing by Basketter et al. (2012) and challenges in developmental neurotoxicity testing in the 21st century versus in vitro opportunities by Smimova et al. (2014) are noteworthy. In recent years, the application of alternatives in testing warfare agents is much appreciated considering the threat of and/or actual terrorist attacks. A variety of such methods have been either validated and/or are being validated. Continued global effort in innovation with reliable assays, instrumentation, and strategies is hoped to produce much anticipated results so one can routinely use such methods in warfare testing and counterterrorism. Regulatory agencies around the globe are working in collaboration with academia and industry, so the stakeholders, the thought, the talk, and the action are on the same page. Meaningful cooperation is sought, as needed, to achieve a common goal of reliable and quality data in our efforts. This kind of collective and collaborative effort and efficient use of resources with funding from multiple bodies, especially the government, will continue to provide impetus in fully achieving the ultimate goal of reducing, refining, and replacing animal use, as applicable, in toxicity and efficacy testing and finding viable alternatives at all times, especially for warfare testing. Going forward, it is believed that with continued innovations in new technologies and instrumentation, ideas and the vision, such as "organ- or organs-on-a-chip" and "human-on-a-chip" as proposed by Lee et al. (2013) and others, would become a reality. Further, with the advancements in science and technology, continued collective efforts are being made by stakeholders in this urgently needed field (PETA, 2019; Mercury News, 2016; Kronman and Moore, 2019). With these advancements, the concerns of excessive use of animals in testing would continue to decrease, yet, quality data be generated with remarkable alternatives.

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Chapter 52

Toxicokinetic aspects of nerve agents and vesicants

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52.1 Introduction

Despite the long history of chemical warfare agents (CWAs), which were first used in large quantities during World War I, vesicants and nerve agents still represent a commonly perceived threat to military and civilian communities. Therefore, effective medical countermeasures and state-of-the-art medical care are mandatory prerequisites to take the threat of poison attacks seriously. Rising interest in the effective treatment of sulfur mustard, and especially nerve agent poisoning, has led national and governmental authorities to continue programs of medical defense against toxicants. The development and continuous improvement of therapies rely on intensified knowledge of provoked pathophysiological effects, toxicology, and toxicokinetics. The latter is important when understanding the reactivity of poisons in organisms at the molecular level. Awareness of predominant biotransformation and efficient elimination processes is essential for the design of a therapeutic regimen for poisoning. For example, the development of protective scavengers is a current challenge in nerve agent defense research.

The toxicokinetic profile of a CWA in mammalian organisms depends on numerous factors, including the nature of the poison, route of exposure, and differences among species. Sophisticated study design, modern technical and analytical monitoring tools, and reliable data from the literature are indispensable quality criteria that should be met when performing toxicokinetic studies. This chapter is focused on this topic.

We first introduce the reader to typical invasion processes of exogenous poisons. The anatomy of exposed areas of the body is discussed, emphasizing interactions with nerve agents and vesicants invading on similar routes following common absorption principles. Afterward, both classes of CWAs are discussed individually. Organophosphorus (OP) nerve agents are distinguished in terms of their physicochemical properties and toxicity, as well as invasion and distribution processes in vivo, which are relevant to toxicokinetic characteristics. Subsequently, special focus is given to the basics of elimination and biotransformation, describing the phenomena of detoxification and concentration decrease at the molecular level. Enzymatic hydrolysis and binding to proteins and enzymes are discussed in comprehensive detail. A few comments on excretion complete this "journey through the poisoned body." The next several sections of the chapter are dedicated to concentration−time profiles, exemplarily selected for typical routes of nerve agent exposure, followed by short introductions to predictive mathematical toxicokinetic models and to most modern bioanalytical methods that allow poison quantification and identification of biotransformation products.

Vesicants, including sulfur mustard and lewisite, are the subject of the second main part of this chapter. Coherences of invasion and distribution are presented, and the major processes of biotransformation and elimination caused by binding to proteins [and more prominently, to deoxyribonucleic acid (DNA)] are discussed. Finally, we make some comments about current bioanalytical approaches. This chapter provides readers with a comprehensive overview of the toxicokinetics of OP nerve agents and vesicants.

52.2 Overview of the invasion processes of CWAs

Toxicokinetics is a subfield of toxicology that studies how, how fast, and to what extent toxicants are absorbed by, distributed in, biotransformed by, and eliminated from the bodies of living organisms. These processes depend

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on multifaceted conditions, such as the species and gender of intoxicated organisms, the physicochemical properties of the toxicant (e.g., hydrophobicity, charge, and molecular weight), their dose and concentration, and their chemical reactivity and stability. Furthermore, the route of exposure is a crucial parameter affecting the effectiveness of incorporation and time for distribution within an organism.

Elaboration of toxicokinetic data of CWAs is essential for designing effective antidotes, improving first aid, and optimizing the therapeutic regimen and medical care. It has to be considered that data obtained from in vitro or in vivo animal studies need careful extrapolation to humans, which at least requires sophisticated mathematical models to consider basic interspecies differences (Langenberg et al., 1997; Sweeney et al., 2006; Levy et al., 2007; Worek et al., 2007).

For controlled toxicological animal studies, the poison is most often administered subcutaneously (s.c.), intravenously (i.v.), or intramuscularly (i.m.), whereas more realistic scenarios of CWA uptake are percutaneous (p.c.) through unprotected skin, by inhalation of aerosols and vapor, or by ingestion (p.o.) of contaminated food and drink. Such exposure events that lead to poison uptake and its distribution in an organism are part of the invasion process, whereas all steps causing a decrease in poison (e.g., elimination by degradation, biotransformation, and excretion) are part of the evasion process. For a better understanding of the pathophysiology and toxicokinetics of CWAs, an overview of the routes of poison incorporation is given in the next section, with a special emphasis on OP nerve agents and vesicants.

52.2.1 Percutaneous uptake by contact with skin

The total skin surface of an adult human is approximately 1.8 m^2, which is very small when compared to the adsorptive surface of the lungs (100 m^2) and the gastrointestinal tract (GIT, 200 m^2; Marquardt et al., 1999). The primary function of the skin is to protect an organism against exogenous compounds present in the external environment. Nevertheless, percutaneous uptake is the predominant route of poisoning by nonvolatile and rather lipophilic agents [e.g., *O*-ethyl *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (VX)], which exhibits low vapor pressure, thus making respiratory incorporation very unlikely except for the inhalation of aerosols (Czerwinski et al., 2006). In the case of vesicant agents, such as sulfur mustard and lewisite, the skin is both a target organ, susceptible to severe local effects, and a pathway for absorption of the agent, leading to its distribution and subsequent systemic effects. The protective skin architecture is provided by a sophisticated and effective barrier built of two main components: the outer epidermis and the underlying inner dermis.

52.2.1.1 Epidermis

The epidermis is composed of various consecutive complex layers, including the stratum corneum (horny layer), stratum lucidum (clear layer), stratum granulosum (granular layer), and stratum germinativum (germinative layer), which itself is subdivided into two parts: stratum spinosum (spinous or prickle layer) and stratum basale (basal layer) (Marquardt et al., 1999). For a general overview of percutaneous poison uptake, we will now restrict the introduction to the horny and germinative layers, which are in this sense the most significant epidermal strata.

The stratum corneum is the upper stratum of the epidermis. It consists of several avascular, stratified cellular layers of dead keratinocytes characterized by a very low water content (5%−10%), thus making the surface hydrophobic and reducing its permeability for polar compounds. This layer, which exhibits the character of a multilayer lipid membrane, is the major barrier hindering hydrophilic substances from invading an organism. Penetration through the horny layer is most often a passive diffusion-controlled process following Fick's law in a good approximation. Because of their lipophilic nature, liquid nerve agents and vesicants can readily penetrate the horny layer, and absorption of liquids is much more effective than that of their corresponding vapors (Blank et al., 1957). VX and SM were shown to form a reservoir in the skin, enabling continuous delayed release of the poison (Hattersley et al., 2008; Rolland et al., 2013). Accordingly, while in contact with the skin barrier nerve agents might also react with skin proteins, thereby forming adducts and detoxifying the agent, as shown for human callus reacting with VX in vitro (Verstappen et al., 2012). To estimate the capability of nerve agents for skin penetration the octanol:water partition coefficient (listed as dimensionless log *P*; see Table 52.1) can be used, which correlates to the lipophilicity of the agent (Czerwinski et al., 1998, 2006). This parameter can also be used to predict the distribution of OP agents in other tissues and blood (Langenberg et al., 1997; Sweeney et al., 2006).

Skin permeation velocity of toxic substances increases with (1) decreasing molecular weight, (2) increasing lipophilicity, (3) increasing area of contaminated skin, and (4) decreasing thickness of contaminated horny layer. The thickness of the stratum corneum varies depending on the area of the body and species. Heavily strained areas (e.g., the sole of the foot, palm of the hand, and insides of the fingers) are protected by a 400−600-μm horny layer in humans, whereas the arms, legs, and body are covered by a barrier of 8−15 μm in thickness. Since the first

TABLE 52.1 Physicochemical properties of VX and G-type OP nerve agents.

Agent	CAS no.	NATO code	MW (g/mol)	Boiling point (°C)	Vapor pressure (mbar)	Water solubility (g/L)	Hydrolysis rate, $\tau_{1/2}$ (h)	log *P* (–)
Cyclosarin	329-99-7	GF	180.2	239	0.059 (25°C)[a]	3.7 (20°C)	n.a.	1.04[b]
Sarin	107-44-8	GB	140.1	158	2.8 (20°C)	Miscible	39 (pH 7.0)	0.30[b]
Soman	96-64-0	GD	182.2	190	0.53 (25°C)	21 (20°C)	45 (pH 6.6)	1.82[b]
Tabun	77–81-6	GA	162.1	237–240	0.049 (20°C)	98 (25°C)	8.5 (pH 7.0)	0.38[b]
VX	50782-69-9	VX	267.4	298	9×10^{-4} (20°C)	30 (20°C)	1000 (pH 7.0)	2.09/0.68[b]

log *P*, octanol:water partition coefficient; MW, molecular weight; n.a., not available; $\tau_{1/2}$, period of half-change for hydrolysis.
[a]*Committee on Gulf War and Health (2004)*.
[b]*Czerwinski et al. (2006)*.
Source: Data are taken from Munro, N.B., Talmage, S.S., Griffin, G.D., 1999. The sources, fate, and toxicity of chemical warfare agent degradation products. Environ. Health Perspect. 107, 933–974 unless otherwise noted.

incidents of chemical warfare in World War I, it was observed that skin areas with a very thin horny layer, in particular the axillae and the scrotum, were most susceptible to the effects of sulfur mustard. Effects in these regions, even though they were rarely exposed to the liquid agent, were severe.

Considering the thickness and structural composition of skin (e.g., the diameter and density of hair follicles, as well as the number of cell layers) is essential for choosing appropriate in vitro models for skin penetration. Pig skin, exhibiting a stratum corneum thickness in the ear of 8.6–28 μm, is similar to human abdominal skin (5.5–40 μm), so it offers a good model to study the agent's permeation as recently shown for VX (Vallet et al., 2008). The best model for p.c. in vivo exposure studies is thought to be the pig after inner ear-skin application of the agent (Chilcott et al., 2003). In contrast to persistent VX, the major fraction (98%) of a small amount of liquid sarin, which was applied p.c. to six human subjects, evaporated very rapidly without passing the skin because of its high volatility (Marrs et al., 2007). In the presence of organic carrier solutes, skin penetration may be enhanced, as shown for VX dissolved in isopropanol, thus doubling the permeation velocity for animal and human skin in vitro (Dalton et al., 2006a,b). In contrast, cooling of exposed skin regions was shown to delay entry of the agent into the circulation, thus representing an optional support for the treatment of cutaneous nerve agent poisoning (Mikler et al., 2011). At the least, experimental conditions should also consider that moisture, heat, and abrasions force the transfer and uptake of permeable CWAs (Blank et al., 1957).

The germinative layer, located under the stratum corneum, consists of living keratinocytes that are responsible for the regeneration and proliferation of skin. It exhibits the highest biotransformation activity of all strata toward endogenous and exogenous substances initiating binding to carboxylesterases (CaE or CarbE). Nevertheless, sorption to cutaneous tissue may form a depot, as demonstrated for sarin (Fredriksson, 1958; Satoh and Hosokawa, 2006), which explains the delayed local fasciculation of muscles observed after s.c. administration of nerve agents (Czerwinski et al., 2006).

52.2.1.2 Dermis

The dermis (corium) consists of connective vascularized tissue composed of collagen, elastic and reticular fibers anchoring sweat and sebaceous glands, and hair follicles. Capillaries pervading the dermis and hypodermis (subcutis, located beneath the dermis) allow systemic distribution of toxic compounds once they have passed the epidermis. Therefore, percutaneously incorporated poison may be directly transported by circulation to any compartment of an organism, or it may be temporarily retained within the skin layers. In addition, it might penetrate with first-order kinetics into subcutaneous tissues and muscles, thus creating a poison depot for delayed release (Wolthuis et al., 1981; Chilcott et al., 2005). If nerve agents are kept in fat tissue, degradation by biotransformation appears rather unlikely, thus maintaining an active release system (Sweeney et al., 2006). Correspondingly, Van der Schans et al. (2003) reported that it took more than 3 h to reach a maximum concentration in blood of lipophilic VX after

percutaneous administration of $1 \times LD_{50}$ to hairless guinea pigs (see Section 52.38).

52.2.2 Respiratory uptake by inhalation

52.2.2.1 Airways and absorption

Gaseous poison or aerosols of toxic substances are incorporated following inhalation and contact with the respiratory tract. This appears to be the predominant route of poison intake for volatile G-type nerve agents (e.g., tabun, sarin, and soman) exhibiting relatively high vapor pressure (Table 52.1). However, uptake by inhalation can also be of significance for nonvolatile agents when they are dispersed as an aerosol. The respiratory tract is composed of three main compartments, distinguishable by the areas and organs involved in the breathing process: (1) the extrathoracic or nasopharyngeal compartment, comprising the mouth, nose, and throat (pharynx); (2) the tracheobronchial compartment, containing the voice box (larynx), windpipe (trachea), and right and left bronchi; and (3) the alveolar or pulmonary compartment of the lung, which includes the bronchioles connecting the bronchi with the lobes of the lung and alveoli. The sacs and cavities of the alveoli allow diffusion-controlled oxygen and carbon dioxide exchange in the blood of pulmonary capillaries (blood−air barrier). This diffusion process follows Fick's law, whereupon the diffusion layer exhibits a thickness of 0.4−2.5 μm composed of surfactant and alveolocapillary membranes. The lung of a healthy adult possesses 300−400 million alveoli spread over a surface of approximately 100 m^2 (Marquardt et al., 1999).

Depending on the physicochemical nature of the toxic gas or aerosol, absorption will take place at different areas of the respiratory tract. Apart from the chemical properties, the size of particles and aerosols will also affect the targeted area. Materials with diameters less than 2 μm will reach the alveoli, whereas larger ones (about 20 μm in diameter) are retained in the upper respiratory areas of the throat and bronchi, preventing gas exchange in the lung.

52.2.2.2 Absorption in the upper respiratory tract

In contrast to highly lipophilic compounds, hydrophilic toxicants, characterized by higher water solubility (e.g., hydrogen chloride and fluorine), are primarily adsorbed by the wet mucosa membranes of the throat and trachea in the upper respiratory tract. This is also the primary area for G agent first-order absorption according to higher water solubility (Sweeney et al., 2006; Marrs et al., 2007). Early studies have shown that 80%−90% of inhaled sarin was readily absorbed by humans when exposed to concentrations ranging from 7 to 43 mg min/m^3 (Oberst et al., 1959, 1968). Slightly lower rates (approximately 70%) were obtained for sarin applied to guinea pigs, dogs, and monkeys (Benschop and de Jong, 2001). More aggressive and reactive agents (e.g., vesicants and pulmonary agents) may cause harm by chemical burns, provoking time-delayed inflammation and scarring in these areas. Langenberg et al. (1998b) found significant effects of sulfur mustard in the upper respiratory tract of guinea pigs, caused by a large fraction of inhaled agent.

The nasal system of guinea pigs is more complex than that of other mammalian species, including humans. In those species, the fraction of agent absorbed or deposited in the upper respiratory system may be smaller. Consequently, the middle and lower respiratory systems are likely to be exposed to a larger amount of the agent.

52.2.2.3 Absorption in the middle respiratory tract

Toxic agents with medium water solubility (e.g., chlorine and sulfur dioxide) reach farther into the middle respiratory tract, being absorbed in the bronchi and its junctions. As a result, elevated excretion of mucus, coughing, and bronchoconstriction will lead to heavy dyspnea. The respiratory tube system from the nose to the bronchioles is lined with ciliated epithelium, allowing transportation of the mucous layer produced by cells of the bronchial system from the lung into the oral cavity. This mechanism, which may be supported by coughing, allows the binding and removal of dust particles, thus protecting the alveoli from deterioration. Chemosensory transient receptor potential (TRP) channels play an important role in this context (Dietrich et al., 2017). Chemosensory TRP channels can be activated by a plethora of reactive chemicals including CWAs (Steinritz et al., 2016; Bessac and Jordt, 2010). Especially TRPA1 and TRPV1 channels were found to be essentially involved in airway chemosensation and reflex control (Bessac and Jordt, 2008). However, once the mucus has reached the oral cavity, spontaneous swallowing might transport poison into the digestive tract of the stomach and gut (i.e., the GIT).

It is the middle respiratory tract that apparently is the most significant target in the case of respiratory exposure to sulfur mustard. Local effects, such as the formation of pseudomembranes which result from cellular debris and a fibrin-rich exudate, may both be life-threatening in the acute phase of illness after poisoning and cause long-term disability (White et al., 2016). The systemic absorption from the middle respiratory tract has never been investigated separately. However, findings from Langenberg's guinea pig model (Langenberg et al., 1998b) suggest that the largest part of inhaled sulfur mustard is deposited in that particular area.

52.2.2.4 Absorption in the alveoli

Several toxic compounds, particularly gases of low water solubility or increased lipophilicity (e.g., carbon monoxide, hydrocyanic acid, nitrogen oxides, phosgene, and further inorganic and organic irritants) reach the alveoli and are absorbed by blood. Aggressive and reactive compounds that are inhaled may damage the epithelial cells of the alveoli, causing the liberation of edema fluid, which fatally prolongs the diffusion layer and minimizes the permeability for oxygen and carbon dioxide, finally leading to unconsciousness or death by asphyxia (toxic lung edema). Only very few hints exist for OP-induced lung edema following a yet-unraveled mechanism of action (Lainee et al., 1991; Delaunois et al., 1992; Niven and Roop, 2004). Similarly, in the case of sulfur mustard exposure, the alveoli are only of minor importance in terms of target organ for both local effects and systemic absorption (Langenberg et al., 1998b).

52.2.2.5 Nose-only exposure model for controlled respiratory uptake in animal studies in vivo

To elucidate the velocity of nerve agent absorption via the respiratory tract, which may take seconds to hours, Langenberg et al. (1998a) designed an apparatus that challenged guinea pigs by nose-only exposure with a constant stream of nerve agent vapor in air held for several minutes. As guinea pigs were not ventilated, artificially sublethal doses of soman and sarin (0.4−0.8 LCt_{50}) were applied, thus enabling the affected respiratory frequency and minute volume to be monitored. A typical concentration−time profile detected for C(−)P(+)-soman after administration of C(±)P(±)-soman is characterized by a discontinuous curvature reflecting concentration increase during absorption and concentration decrease by elimination (see the section "Respiratory uptake (nose-only model)," later in this chapter). For more detailed technical data on the nose-only apparatus, see Langenberg et al. (1998a,b) and Benschop and de Jong (2001). The same researchers also used this apparatus in subsequent experiments to investigate the respiratory uptake of vesicant agents, results of which are discussed in Section 52.39.

52.2.3 Gastrointestinal uptake by ingestion

When an animal drinks poisoned liquids or ingests contaminated food, toxicants will be incorporated and directly transferred from the mouth, through the gullet (esophagus), and into the stomach and bowels (intestine) representing the GIT, where transfer into the circulation occurs. The physiological function of the digestive tract includes intake, breakdown, transport, and digestion of food and creation of waste (excrement). Food ingredients, as well as toxicants and their digested (biotransformed) forms, are either absorbed through the walls of the intestine and enter circulation or are eliminated in feces. The surface of the small intestine of an adult human covers more than 200 m^2 and is made up of 4−5 million tiny, fingerlike projections (villi and microvilli) covering the surface of the mucous membrane. In contrast, the resorbing areas of the large intestine (0.5−1 m^2), stomach (0.1−0.2 m^2), rectum (0.04−0.07 m^2), and oral cavity (0.02 m^2) are explicitly smaller due to lack of villi; therefore, they are of minor importance for poison uptake. Uptake by diffusion through lipid layers (hydrophobic molecules) and pores (small hydrophilic molecules) of the intestinal mucosa (following Fick's law) are the most common processes. Nevertheless, facilitated diffusion and active transport, based on affinity binding of the toxicant to carrier molecules, might also occur (Marquardt et al., 1999). The processes of pinocytosis and phagocytosis are unlikely with small CWAs. Following intestinal uptake into the circulation, toxicants are directly transported to the liver, where further biotransformation by cytochrome P450 enzymes may happen. This is of essential relevance for toxification of OP pesticides, including parathion, chlorpyrifos, diazinon, and dimethoate, which are transferred into their more toxic oxon derivatives (Butler and Murray, 1997; Furlong, 2007). The liver and intestine are supposed to play very important roles in the elimination of free soman, as deduced from rabbit studies at high soman dosing (Li et al., 2002).

In contrast, for sarin, it has been shown that the kidney is more important for detoxification than the liver (Little et al., 1986). However, only very limited research efforts have been undertaken to characterize this route of poison uptake for nerve agents (Sim et al., 1971; Sidell and Groff, 1974). Very high lethal oral doses were reported for tabun (rabbit: 16,300 μg/kg; rat: 3700 μg/kg; dog: 200 μg/kg) and sarin (rat: 550 μg/kg), indicating extensive hydrolysis in the GIT (Marrs et al., 2007). Very few investigations of the toxicokinetics of vesicants after ingestion have been conducted. Nevertheless, this route of exposure is supposed to result in significant systemic absorption of an agent. The rationale behind this apparent "blind spot" of toxicokinetic investigation might be the rarity of gastrointestinal exposure during conditions of military operations. Moreover, should a GIT exposure occur, the local, rather than the systemic, effects would be life-threatening. See Section 52.17 for an in-depth discussion. In contrast, ample work has been done with OP pesticides to address the troubling statistics of more than 500,000 deaths per year worldwide caused by accidental and suicidal ingestion (Eddleston et al., 2005, 2008a,b).

52.2.4 Uptake by intravenous injection

Intravenous uptake of CWAs is highly unlikely for realistic exposure scenarios, except perhaps for the contamination of open and bleeding wounds. Nevertheless, numerous scientific studies investigating toxicity and therapeutic treatment of CWAs have made use of this route of administration. The rationale behind this design is to constitute a defined amount of poison in blood, which is immediately systemically distributed by the circulation to the target compartments under conditions of 100% bioavailability. Therefore, i.v. studies are undoubtedly relevant to elaborate systemic toxicity and to characterize the impact on the whole organism. It was shown that sarin was distributed in the central nervous system (CNS) within 20−30 s after i.v. administration to mice (Waser and Streichenberg, 1988). Therefore, poison acts without delayed uptake caused by diffusion or permeation through skin, tissue, and organs, which would increase the extent of degradation and hydrolysis by biotransformation. The latter processes reduce the amount of toxic agents and hamper making a direct correlation to poison concentrations in blood. As demonstrated by Van der Schans et al. (2003) only 2.5% bioavailability was observed in guinea pigs, resulting from a 7-h permeation period after percutaneous administration of VX.

As outlined in previous sections, the amount of incorporated poison and its fraction interacting with target molecules to induce more or less specific toxicological effects may vary drastically, even under controlled conditions of administration. However, monitoring of quantitative concentration−time profiles of the original poison and its biotransformation products is needed to elucidate the kinetic behavior and to unravel pathophysiological mechanisms. Due to the broad range of such essential variations causing a number of uncertainties, many in vivo and in vitro studies have been performed during the last 30 years of sophisticated research on protection against OP compounds (OPCs) and blister agents. The following sections summarize the most important consolidated findings underlining the current status of toxicokinetics of the most prominent CWAs: nerve agents and vesicants.

52.3 Nerve agents

52.3.1 OPCs as nerve agents

For important background information addressing the history, synthesis, basic chemical data, decontamination, toxicity, military use, and political relevance of nerve agents, we refer readers to a number of textbooks, reports, and monographs that go beyond the scope of this toxicokinetic contribution (e.g., Koelle et al., 1963; Franke, 1977; Munro et al., 1999; Augerson, 2000; Committee on Gulf War and Health, 2004; Langford, 2004; Marrs et al., 2007; Richardt and Blum, 2008). Nevertheless, in this section, selected chemical and physicochemical properties are considered that are of importance for understanding the toxicokinetic behavior of OPCs.

Well-known representatives of nerve agents are cyclosarin (*o*-cyclohexyl methylphosphonofluoridate; GF), sarin (isopropyl methylphosphonofluoridate; GB), soman (1,2,2-trimethylpropyl methylphosphonofluoridate; GD), tabun (ethyl dimethylphosphoramidocyanidate; GA), VX (*S*-2-diisopropylaminoethyl *o*-ethyl methylphosphonothioate), and Russian VX [*o*-isobutyl *S*-(*N*,*N*-diethylaminoethyl)methyl phosphonothioate; RVX, VR] (see Table 52.1 and Fig. 52.1). Apart from these substances, a large number of additional OP toxicants with high structural similarity have been seen on experimental and laboratory levels, especially when pesticides for civilian use are included. However, the abovementioned compounds are the most prominent nerve agents in the literature.

This fact is obviously due to political instructions for national security and defense programs that consider historical development, large-scale production, and intended military use and focus on agents that most likely would be relevant chemical threats. Soman, for example, was produced in large amounts during the Cold War by the Soviet Union in particular, thus menacing the Western world. Therefore, toxicokinetic studies on nerve agents are mainly restricted to sarin, soman, and VX. In addition, following United Nations (UN) inspections in Iraq in the 1990s, which revealed the weaponizing of cyclosarin, some studies on the toxicokinetic properties of cyclosarin were also conducted.

52.3.2 Physicochemical properties

If they are present in a highly pure state, all nerve agents are colorless and odorless liquids characterized by different vapor pressures, thus causing either very rapid evaporation with increased inhalational risk (e.g., G-type agents) or relatively high resistance abating p.c. absorption (e.g., V-type agents and cyclosarin; see Table 52.1). Therefore, visual or sensory recognition of these substances by smell or taste is nearly impossible.

52.3.2.1 Water solubility

Water solubility is a crucial parameter affecting the toxicological potency of a compound. Solubility of nonhydrolyzed nerve agents is fundamentally determined by the hydrophobicity and extent of organic substituents. On one hand, hydrophilic compounds exhibiting great water solubility are less effectively absorbed by skin in the absence of organic carrier solutes, but they are easily distributed once they have reached aqueous biological fluids.

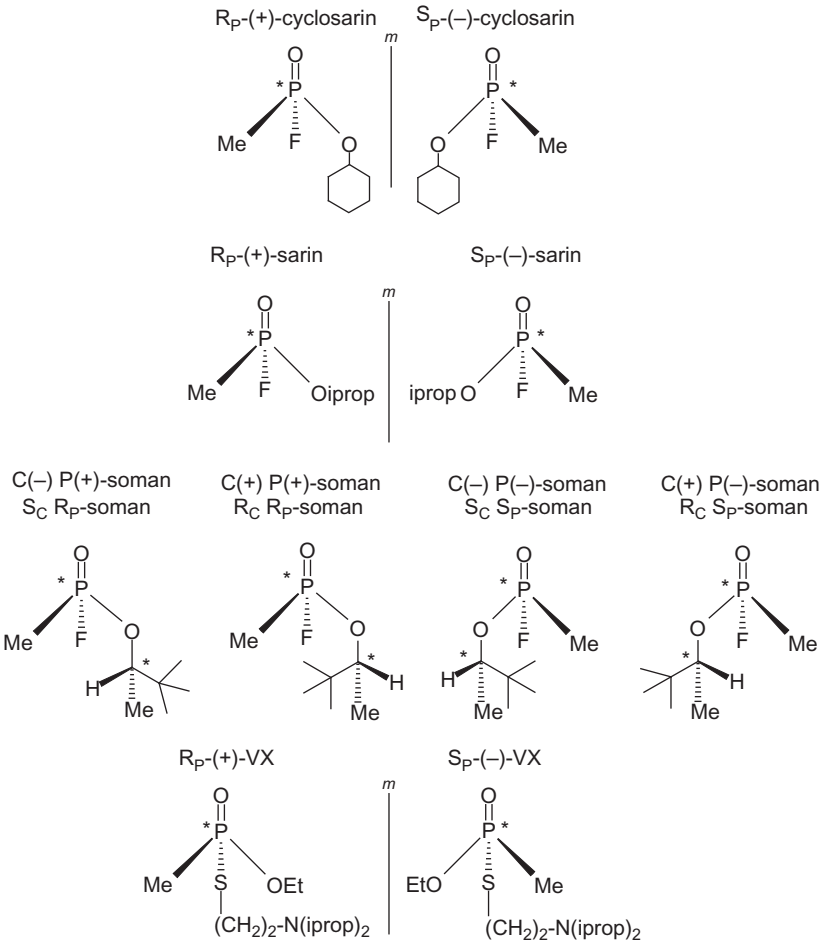

FIGURE 52.1 Structures of stereoisomeric OP nerve agents. Chirality was assigned according to the rules of Cahn, Ingold, and Prelog (Cahn et al., 1966; Prelog and Helmchen, 1982) considering oxygen of the PO bond with minor priority compared to alkoxy substituents (Quin, 2000). Empirically found opticity is, according to Benschop and de Jong (1988), correlated to compounds obtained from stereoselective synthesis (Li et al., 2001). Chirality emerged as a crucial parameter for toxicokinetic properties. S_P-isomers (P(−)-forms) of illustrated G agents (cyclosarin, GF; sarin, GB; soman, GD) are characterized by higher toxicity combined with higher stability against hydrolyzing mammalian wild-type enzymes as their corresponding RP-isomers, P(+)-forms (Table 52.3).

Therefore, skin penetration of less lipophilic sarin (miscible in water) is not favored, whereas nerve agents of significant lipophilicity with low water solubility (VX: 30 g/L; GF: 3.7 g/L; see Table 52.1) penetrate skin and other hydrophobic biological membranes and mucosa unhampered (Winkenwerder, 2002). Nerve agent transfer into blood and its systemic distribution are limited, potentially provoking accumulation in fatty tissue. Nevertheless, noncovalent binding to carrier proteins of circulation (e.g., γ-globulin and albumin) may support systemic transport of not very water-soluble compounds in blood, as would be expected for VX (Vallet et al., 2008), and as commonly known for endogenous fatty acids or exogenous drugs (John and Schlegel, 1999; Li et al., 2007; Weiss et al., 2008). Table 52.1 denotes corresponding measures of solubility in water.

52.3.2.2 Octanol:water partition coefficient

The lipophilicity of nerve agents and their expected partition behavior are characterized by the octanol:water partition coefficient (log *P*). This parameter is often used as an estimate for the tendency of the toxicant to bioaccumulate in an organism. Furthermore, it is helpful to predict the penetration of skin and distribution between tissue and blood (Poulin and Krishnan, 1995; Czerwinski et al., 1998, 2006). The log *P* for the more polar sarin was determined to be 0.30, whereas this measure for lipophilic VX is 2.09, which documents a 50-fold higher lipid solubility. However, this ratio might be slightly different when considering the inconsistency of data from the literature. Table 52.1 summarizes the log *P* values of selected nerve agents.

52.3.2.3 Hydrolysis

In aqueous media, nerve agents undergo nonenzymatic hydrolysis, which is accelerated by very acidic and basic pH values (Franke, 1977). Hydrolysis primarily substitutes the reactive leaving group of the OPC for a hydroxy group, thus making the molecule more soluble (Fig. 52.2). After cleavage of diisopropyl ethyl mercaptoamine (DESH) from the phosphorus atom of VX (Fig. 52.1), the remaining ethyl methylphosphonic acid exhibits a water solubility of 180 g/L, which is six times higher than VX itself (Munro et al., 1999). Depending on pH, the

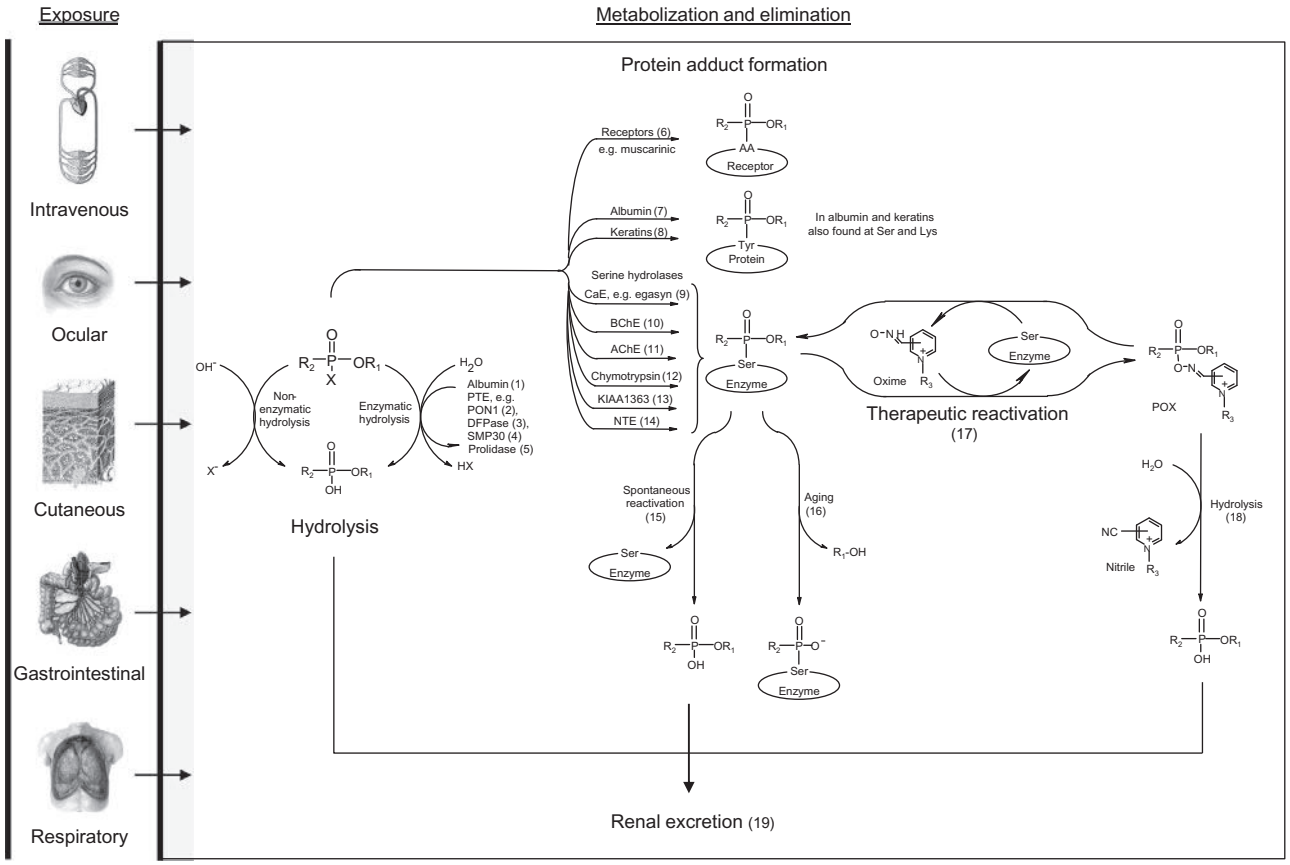

FIGURE 52.2 Elemental steps of toxicokinetics of OP nerve agents in mammalian organisms. OP nerve agents are incorporated following different routes of exposure. Whereas G agents are predominantly taken up by the respiratory tract via inhalation, V agents mainly follow percutaneous invasion. Once the poison has reached the circulation, it is distributed systemically, causing poisoning of the CNS and PNS by inhibition of AChE. Several processes of biotransformation (e.g., enzymatic and nonenzymatic hydrolysis) and elimination (e.g., formation of adducts by binding to proteins and multiple serine esterases, followed by marginal spontaneous reactivation and more prominent aging) reduce the amount of circulating poison. Therapeutic causal intervention by oximes reactivates cholinesterases under liberation of a toxic phosphoryloxime intermediate (POX), which itself undergoes immediate hydrolysis. Hydrolyzed nerve agents emanating from these chemical conversions are excreted by the kidney more prominently than by liver. AA, amino acid; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CarbE, carboxylesterase; DFPase, diisopropyl fluorophosphatase; KIAA1363, acetyl monoalkylglycerol ether hydrolase; Lys, lysine; NTE, neuropathy target esterase; PON1, paraoxonase 1; POX, phosphoryloxime; PTE, phosphotriesterases; $R_{1/2}$, organic substituents, e.g., methyl, cyclohexyl, isopropyl; Ser, serine; SMP30, senescence marker protein 30; Tyr, tyrosine; X, nucleophilic leaving group; e.g., F, CN, (CH2)2-N(iprop)2. (1) Vilanova and Sogorb (1999); Manoharan and Boopathy (2006); Li et al. (2007); (2) Billecke et al. (2000); Amitai et al. (2006); Furlong (2007); (3) Blum et al. (2006); Nordgren et al. (1984); (4) Kondo et al. (2004); (5) diTargiani et al. (2010); (6) Silveira et al. (1990); (7) Black et al. (1999); Li et al. (2007, 2008); Grigoryan et al. (2009); John et al. (2010); (8) Grigoryan et al. (2009); Schopfer et al. (2010); Verstappen et al. (2012); (9) Maxwell and Brecht (2001); Satoh et al. (2002); Fujikawa et al. (2005); (10) Bartling et al. (2007); Kolarich et al. (2008); (11) Benschop and de Jong (2001); Aurbek et al. (2006); (12) Casida and Quistad (2005); (13) Nomura et al. (2008); (14) Gordon et al. (1983); Costa (2006); (15) Worek et al. (2005); Aurbek et al. (2006); (16) Worek et al. (1998, 2005); Bartling et al. (2007); (17) Sidell and Groff (1974); Kiderlen et al. (2005); Aurbek et al. (2006); Bartling et al. (2007); (18) Kiderlen et al. (2005); (19) Waser and Streichenberg (1988); Shih et al. (1994); Minami et al. (1997).

alkoxy-group of VX might also be cleaved from the phosphorus atom. Hydrolysis of the reactive electronegative leaving group, which is essential for primary toxicity [inhibition of acetylcholinesterase (AChE)], deactivates the molecule, reducing its toxicity dramatically. The major hydrolysis product of sarin is isopropyl methylphosphonic acid (IMPA), which is about 10,000 times less toxic than its precursor when administered orally to rats (Munro et al., 1999). IMPA is also the most prominent biotransformation product of sarin produced in vivo (Little et al., 1986). The dramatically increased rate of hydrolysis under acidic conditions is presumably the most important reason for extraordinarily high lethal dose (LD_{50}) values found in laboratory animals after oral administration of nerve agents (Marrs et al., 2007). Table 52.1 summarizes hydrolysis rates expressed as periods of half-change in aqueous solutions of nerve agents near neutral pH.

52.3.2.4 Chirality

Typical production batches of nerve agents formerly intended for military use are mixtures of enantiomers obtained from nonchiral synthesis (Fig. 52.1). Sarin, cyclosarin, tabun, and VX consist of mixtures of two enantiomers; each of which differs in the chirality at the central phosphorus atom. This enables the rotation of linearly polarized light clockwise [P(+)-enantiomers] or anticlockwise [P(−)-enantiomers; see Fig. 52.1]. In contrast, chirality of soman appears more complex based on two chiral centers, which reside at the phosphorus atom, P(+) and P(−), and additionally in the pinacolyl moiety, C(+) and C(−). Hence, soman occurs in four stereoisomeric conformations as two pairs of diastereomers: P(+)C(+), P(+)C(−), P(−)C(+), and P(−)C(−); (Fig. 52.1). To denominate stereoisomers of nerve agents, the experimentally found rotational direction of light is typically provided, whereas assignments according to the R and S nomenclature are rare. Therefore, Fig. 52.1 summarizes both absolute configurations and related optical activities of the most common nerve agents. Chirality was assigned according to the established rules of Cahn, Ingold, and Prelog (Cahn et al., 1966; Prelog and Helmchen, 1982), considering oxygen of the P═O bond with minor priority to alkoxy substituents (Quin, 2000). Empirically found opticity is, according to Benschop and de Jong (1988), correlated to compounds obtained from stereoselective synthesis (Li et al., 2001).

It is well known that chirality of OP nerve agents causes significantly differing toxicological properties determining poison elimination by hydrolysis and kinetics of enzyme inhibition (Benschop and de Jong, 1988). Therefore, special attention should be paid to the corresponding stereoisomers when elaborating toxicokinetics. A number of studies took care of these conformational differences by administration of pure stereoisomers (Benschop and de Jong, 2001) or by enantioselective detection and quantification of poison molecules (Spruit et al., 2001; Li et al., 2003a,b; Van der Schans et al., 2003; Reiter et al., 2007, 2008, 2011; Yeung et al., 2007; Tenberken et al., 2010). Different techniques were established to produce pure (or at least enriched) enantiomers of nerve agents, for example, (1) synthesis by use of chiral adducts subjected to reactions of defined stereochemical outcome; (2) fractional crystallization, chromatographic separation, and isolation of mixed enantiomers; (3) stereoselective binding to serine esterases (e.g., α-chymotrypsin); and (4) stereoselective enzymatic hydrolysis by phosphorylphosphatases (chemoenzymatic preparation; Benschop and de Jong, 2001; Li et al., 2001).

Stereoselective enzymatic degradation of nerve agents is also a current issue in developing both novel noncorrosive decontamination systems and new therapeutics using recombinant mutated enzymes optimized for fast and exhaustive hydrolysis of most toxic isomers (Tsugawa et al., 2000; Li et al., 2001; Ghanem and Raushel, 2005; Furlong, 2007; Blum and Richardt, 2008).

52.3.3 Toxicity

OPCs, especially nerve agents, represent a class of highly reactive compounds undergoing nucleophilic substitution of their leaving group [e.g., F, CN, S-$(CH_2)_2$-N(iprop)$_2$; Fig. 52.1] by nearly irreversible coupling to nucleophiles (e.g., strongly polarized hydroxyl groups in amino acid side chains or OH functions in aqueous media; Fig. 52.2). Toxic effects are mainly due to derivatized enzymes which were subjected to this reaction.

Although most toxicological studies were performed as animal studies, some data about humans exist, which were obtained from military volunteers exposed to nonlethal doses of sarin (NRC, 1982, 1985), accidentally intoxicated industrial workers (Duffy et al., 1979), and poisoned civilians affected by terrorist attacks in Tokyo in 1995, Matsumoto in 1994, and Osaka (Morita et al., 1995; Okumura et al., 1996; Tsuchihashi et al., 1998). For detailed data on these events, see the reports of the Committee on Health Effects Associated with Exposures During the Gulf War (2000).

52.3.4 Inhibition of AChE

Phosphorylation of the OH moiety of serine residue, being part of the catalytic triad in the esteratic center of AChE, represents pathophysiologically the most important reaction, resulting in enzyme deactivation. Inhibition of AChE was proved to be the predominant major reaction in vivo, which causes death within minutes in mammals, insects, and other species depending on acetylcholine (ACh)-mediated signal transduction. Maxwell et al. (2006) found compelling arguments that inhibition of AChE by nerve agents is the primary mechanism of OP toxicity. They correlated the median LD_{50} of highly toxic nerve agents determined from rats after s.c. administration with the corresponding bimolecular rate constants of AChE inhibition determined in vitro, using a probit model for interpreting the mathematical relationship.

AChE is present in the nervous system, where it is most important for toxic effects, and on the surface of red blood cells (RBCs), where its biological function is still unknown. Inactivation of AChE hinders the degradation of the neurotransmitter ACh in the synaptic cleft, which is of major importance for regulation of presynaptic and especially postsynaptic effects. Rising ACh concentrations cause permanent overstimulation of muscarinic (subtypes m1−m5) and nicotinic receptors of effector cells, leading to cholinergic crisis and, ultimately, death. Clinical

symptoms of poison-induced AChE inhibition include (1) muscarinic effects (e.g., miosis, bradycardia, increased secretion of urine, saliva, tears and sweat, bronchoconstriction, and increased gastrointestinal motility); as well as (2) nicotinic effects (e.g., muscular weakness, twitching and tremors, elevated blood pressure, and tachycardia); and (3) central effects (e.g., headache, impaired memory and alertness, anxiety, insomnia, and, most important, respiratory depression and paralysis). Death is caused by respiratory failure, as elicited by flaccid paralysis of respiratory muscles, and bronchoconstriction, together with increased bronchial secretion and central respiratory depression (Costa, 2006). Liquids or vapors from these agents can cause death within minutes after exposure. Table 52.2 summarizes the LD_{50} values for different species and routes of administration for the most

TABLE 52.2 Acute lethality of OP nerve agents.

	LD_{50} (μg/kg)			
	Sarin	**Soman**	**Tabun**	**VX**
Intravenous				
Human	14[a]		14 (LD_{Lo})[b]	1.5 (TD_{Lo})[c]
Rat	45–63[b]	44.5[d]	70[b]	7–10[b]
Mouse	83[e]	35[d]	150[c]	20[e]
Guinea pig		27.5[e]		
Rabbit	15[c]		63[c]	
Percutaneous				
Human	$24–28 \times 10^3$[b]	18,000 (LD_{Lo})[c]	$14–21 \times 10^3$[b]	86 (LD_{Lo})[c]
Rat	2500[b]		18,000[d]	
Mouse	1080[d]	7800[c]	1000[d]	
Guinea pig	8750[f]	9930[f]	25,840[f]	34[f]
Rabbit	925[d]		2500[c]	
Subcutaneous				
Human				30 (LD_{Lo})[c]
Rat	103–108[a]	70–165[b]	162[b]	12[c]
Mouse	170[a]	156[e]	250[c]	22[d]
Guinea pig	30[c]	24[d]	120[c]	8.4[d]
Rabbit	30[c]	20[d]	375[c]	14–66[d]
Respiratory[g]				
Human	50–100[a]	70 (LD_{Lo})[c]	150 (LD_{Lo})[c]	5–15[h]
Rat	80–300[a]		30.4[d]	
Mouse	240–380[a]	33.3[c]	0.5[c]	
Guinea pig	100–200[a]		197[c]	
Rabbit	75–144[a]		84[c]	

LD_{Lo}, lethal dose, low: the minimum amount of a chemical which has shown to be lethal to a specified species; TDLo, lowest toxic dose. Data do not consider chiral distinctions.
[a] *Winkenwerder (2002).*
[b] *Subcommittee on Chronic Reference Doses for Selected Chemical Warfare Agents, National Research Council (1999).*
[c] *Maynard et al. (1992).*
[d] *ToxNet, Toxicology data network.*
[e] *Benschop and de Jong (2001).*
[f] *Czerwinski et al. (2006).*
[g] *Given as LCt_{50} (mg min/m^3).*
[h] *Augerson (2000).*

common nerve agents. The relative lethality of these substances, determined in animal studies, is as follows, listed in descending order: VX, soman, cyclosarin, and tabun (Sidell and Borak, 1992).

Local irritations do not occur except by fasciculation of underlying muscles after percutaneous uptake or miosis caused by excessive stimulation of muscarinic receptors on the papillary sphincter muscles, resulting from ocular exposure (Sidell and Borak, 1992; Dabisch et al., 2008). However, clinical symptoms related to massive restraints in motoric and respiratory abilities require the reduction of AChE activity by more than 70%, as deduced by Thiermann et al. (2005) from murine diaphragm experiments. Accordingly, acute cholinergic syndromes in humans were not observed until the RBC AChE activity was inhibited by 75%–80% (Sidell and Borak, 1992). Clinical symptoms may depend on the gender of the animal, as recently documented for the extent of miosis after vapor exposure to soman, cyclosarin, and VX (Dabisch et al., 2008). Male rats were approximately three times less sensitive than female rats, whereas miniature pigs show the reverse effect. This phenomenon refers to different activities of ocular AChE and butyrylcholinesterase (BChE; Dabisch et al., 2008).

52.3.5 Additional targets with potential clinical relevance

As binding to proteins other than AChE reduces the amount of free OP poison, these alternative targets are to be considered when discussing toxicokinetic behavior. Albumin (Black et al., 1997a,b; Li et al., 2007, 2008; Williams et al., 2007; John et al., 2010), receptor/channel complexes (Pope, 1999), muscarinic and nicotinic ACh receptors (Bakry et al., 1988; Silveira et al., 1990), and other secondary serine hydrolase targets were shown to be chemically modified by OPCs; changing their functionality into potential pathophysiological situations by affecting noncholinergic mechanisms (Duysen et al., 2001; Casida and Quistad, 2005). In general, chemical modification of any protein and enzyme requires very high OPC concentrations that are far beyond the lethal dose of nerve agents, thus being of only minor relevance for acute poisoning scenarios (Pope, 1999). In contrast, elaboration of toxicity of the much-less-toxic OP pesticides is more and more focused on these additional targets; investigating, for example, genetic susceptibility, developmental toxicity and neurotoxicity, delayed neurotoxicity, and organophosphate-induced delayed polyneuropathy (OPIDP), a distal sensorimotor axonopathy (Costa, 2006; Balali-Mood and Balali-Mood, 2008). OPIDP is associated with OP-inhibited enzyme neuropathy target esterase (NTE), which undergoes an essential aging process of phosphylated NTE (elimination of an organic substituent from the central phosphorus atom, as shown in Fig. 52.2; Costa, 2006). However, Gordon et al. (1983) demonstrated that, despite NTE inhibitory potency of the nerve agents tabun, soman, and VX, no OPIDP was induced. Nerve agent concentrations inhibiting half of the NTE activity were about three orders of magnitude higher (micromolar range) than for AChE (nanomolar range).

Using microarray technology, Gao et al. (2013) performed a toxicogenomic study on neural cells that had been exposed to VX. A huge number of affected gene expressions relevant in numerous physiological and pathophysiological processes were identified, indicating a broad variety of targets for potential harm. Specific long-term changes in the brain were also observed after exposure to nerve agents (Zhu et al., 2010; Spradling et al., 2011; Oswal et al., 2013). In addition, epigenetic changes were found in cell culture models after paraquat exposure (Song et al., 2011). The aerotoxic syndrome in humans is discussed as possibly being associated with the incorporation of OP tri-O-cresyl-phosphate (TOCP) as an additive in jet hydraulic and engine fluids (Carletti et al., 2013). However, the mechanism causing signs and symptoms is still unknown.

52.3.6 Elemental steps of nerve agent toxicokinetics

Although toxicological characterizations in terms of mean acute lethal doses for different species are available for all nerve agents, detailed and extensive toxicokinetic data are rare in literature for most of them, with the exception of soman and sarin (Benschop and de Jong, 2001). For VX, and in particular GF, only a very limited extent of data is available (Van der Schans et al., 2003; Reiter et al., 2007).

52.3.6.1 Invasion

Supplementary to the more common discussion on invasion processes given in the introductory overview (see Section 52.2), we now present some recent findings on skin penetration models. Nerve agents are readily absorbed through the skin, eyes, lungs, and GIT. Depending on individual vapor pressures of nerve agents, different routes of poison uptake are preferred. G agents (such as sarin, soman, and tabun) are very volatile, limiting percutaneous uptake due to significant evaporation from the skin (approximately 98% for sarin). In contrast, VX exhibits high persistency due to its vapor pressure being 3000 times lower than that of sarin (Marrs et al., 2007; see Table 52.1). Therefore, percutaneous uptake is most prominent for V agents, characterized by an absorption rate of at least 600 $\mu g/cm^2/h$, as shown for VX after

inner ear-skin droplet application of 2 LD_{50} (Fig. 52.2; Chilcott et al., 2005). Nevertheless, VX penetration through skin is characterized in vitro by a significant lag time of at least 1 h and a moderate penetration rate of about 1%−2% per hour (Vallet et al., 2008). These data are in accordance with early studies on human subjects demonstrating that, 3 h after percutaneous exposure of VX, only 0.4%−0.6% was incorporated (Vallet et al., 2008). In vitro studies using guinea pig and human skin, as well as dermatomed, abdominal skin from domestic pigs, revealed significant differences in VX permeability. The highest permeability was observed for the skin of the guinea pig, whereas no significant differences in penetration kinetics were found for human skin and skin taken from a pig's flank (Dalton et al., 2006a,b). Therefore, pig skin may serve as an appropriate in vitro model for human skin (Vallet et al., 2008), as already established in pharmaceutical research using Franz-type diffusion cells (Franz, 1975; Simonsen and Fullerton, 2006). To predict in vivo human VX absorption via skin, full-thickness human abdominal skin has also been demonstrated to be appropriate (Vallet et al., 2008).

52.3.6.2 Distribution

Once nerve agents have penetrated the blood, systemic distribution, including crossing of the blood−brain barrier (BBB), causes toxicity within the CNS and peripheral nervous system (PNS). In mouse and rat studies, it has been shown that within 1 min after sublethal single-dose i.v. administration of sarin, the nerve agent was present in many other compartments (e.g., the diaphragm, heart, lung, and brain) and in much higher concentrations in the plasma, liver, and kidney (Little et al., 1986; Waser and Streichenberg, 1988). Similar results were observed for VX (Chilcott et al., 2005) and soman, which was also found in cerebrospinal fluid with 100% bioavailability after i.v. bolus injection into pigs (Göransson-Nyberg et al., 1998; Augerson, 2000). For a mathematical description, Langenberg et al. (1997) calculated related tissue/blood partition coefficients for the distribution of soman in guinea pigs, revealing a measure of approximately 2 for liver and 1.1 for kidney; whereas the values for the lung and brain were calculated as 0.5. Sarin present in the brain did not severely inhibit AChE activity in the cortex (60%), striatum (40%), and hippocampus (56%) within 24 h (Whalley and Shih, 1989). In contrast, soman has caused more severe inhibition in these three areas (83%−99%), lowering the synaptosomal sodium-dependent, high-affinity choline uptake (SDHACU) within the first 4 h in the hippocampus; whereas from 2 to 24 h after exposure, SDHACU increased in the striatum (Whalley and Shih, 1989). The differences are thought to be due to different aging rates and different AChE-inhibiting potencies. However, the brainstem and midbrain were influenced by neither sarin nor soman. It is assumed that active sites of the brain are affected primarily due to increased metabolic action, vasodilation, and increased blood flow in these regions (Scremin and Jenden, 1996). In contrast, 50% inhibition of AChE activity in brain was associated with death or serious signs of toxicity in mice after subcutaneous exposure (Duysen et al., 2001).

The presence of sarin, soman, and VX in the brain demonstrates the necessity for antidotes, especially AChE reactivators, to be capable of passing the BBB, representing a current scientific challenge (Lorke et al., 2008; Okuno et al., 2008).

Concentrations of sarin found in different tissues were decreased by 85% within 15 min after exposure (Committee on Gulf War and Health, 2004). Apart from active sarin, its inactivated biotransformation product, IMPA, was found in these tissues in a predominant ratio, indicating rapid in vivo biotransformation. This finding is discussed in the next section. Soman was mainly accumulated in the lung after s.c. challenge of rats (Shih et al., 1994) and disappeared from blood and liver 2 min after i.p. administration of $0.75 \times LD_{50}$ to mice (Nordgren et al., 1984). In contrast, VX is more persistent in vivo than sarin or soman, causing delayed systemic distribution after s.c or p.c. administration. This provoked maximum concentrations of VX in blood several hours after p.c. exposure in guinea pigs (Van der Schans et al., 2003).

As outlined in Section 52.13, the toxicological mechanism of action of nerve agents is based on the chemical reactivity of the nucleophilic leaving group. Therefore, biotransformation in terms of degradation by hydrolysis and binding to proteins determines bioavailability and elimination processes, regulating toxicity.

52.3.6.3 Biotransformation and elimination

Enzymes from plasma and tissue are mainly responsible for hydrolysis of OPCs producing derivatives of phosphoric and phosphonic acids characterized by high water solubility and nearly no toxicity. In vivo studies in mice have shown that, within 1 min after injection, 50% of sarin was rapidly biotransformed, generating both free hydrolyzed IMPA and bound IMPA attached to esterases by phosphorylation as predominant in plasma (Little et al., 1986). In contrast, V-type agents are more stable against enzymatic hydrolysis, but may undergo additional biotransformation pathways, including oxidation of nitrogen, sulfur, or both (Van der Schans et al., 2003). Due to the slow reaction velocity of nonenzymatic hydrolysis, this process is of minor importance for elimination kinetics of nerve agents under physiological conditions near neutral pH. As listed in Table 52.1, periods of half-life for nerve agent hydrolysis range from

approximately 9 h for tabun to 6 weeks for VX. In contrast, the velocity of enzymatic hydrolysis in blood is much faster, defining the rate-determining step for poison elimination.

52.3.7 Enzymatic hydrolysis

Enzymes that hydrolyze OPCs cleave the reactive leaving group of OPCs (e.g., F or CN) from the central phosphorus atom, initiating nucleophilic substitution by a hydroxyl group. Hydrolyzing enzyme activity is present in plasma, and to a much higher extent in kidney and liver, enabling the removal of toxicants from the circulation (Sweeney et al., 2006). Early studies using an isolated hydrolyzing rat liver enzyme demonstrated degradation efficacy for nerve agents in the following order: sarin, soman, and then tabun (Little et al., 1989).

Biotransformation products deactivated by hydrolysis are nearly nontoxic and easily eliminated from the organism via renal excretion (Munro et al., 1999). Despite this capability to hydrolyze OPCs, the original physiological functions and substrates of different enzymes vary significantly, not allowing assignment to a specific class of enzymes (Fig. 52.2). The following section presents the most important enzyme systems relevant for nerve agent biotransformation.

52.3.7.1 Phosphotriesterases

Based on historical development, different (and sometimes inconsistent) nomenclatures were used to assign enzymes that lead to the degradation of OPs. Meanwhile, the International Union of Biochemistry has introduced systematic rules and numbering to classify the relevant enzymes in the following way: hydrolases (group 3), which cleave ester links (group 3.1), that may represent carboxylester hydrolases (group 3.1.1) or phosphoric triester hydrolases, phosphotriesterase (PTE) (group 3.1.8). The latter group contains aryldialkylphosphatase (EC 3.1.8.1) and diisopropyl fluorophosphatase (EC 3.1.8.2; Vilanova and Sogorb, 1999). As these enzymes catalyze substrate cleavage without self-inhibition, they are representatives of A-esterases.

- Paraoxonase 01 (PON1): Paraoxonase (PON1, EC 3.1.8.1; formerly EC 3.1.1.2) is a calcium-dependent, liver-expressed P450 PTE belonging to the class of A-esterases with broad substrate specificity toward various lactones and esters, which is present in liver and plasma associated with high-density lipoprotein particles (Vilanova and Sogorb, 1999). P450 isozymes and variants are well known for their dual role in OP biotransformation. On one hand, they bioactivate less toxic phosphorothioates to their highly toxic oxon derivatives via monooxygenase activity. On the other hand, they hydrolyze and detoxify OP insecticides, such as paraoxon, chlorpyrifos, and diazinon by dearylation, as well as nerve agents (e.g., sarin and soman) by defluorination (Davies et al., 1996; Kiderlen et al., 2005; Furlong, 2007; Fig. 52.2). The original physiological function of PON1 is involved in inactivation of toxic products produced by lipid oxidation (Dragonov and Du, 2004). In addition, PON1 hydrolyzes OPCs, such as sarin, cyclosarin, tabun, and soman, thus accomplishing enzymatic protection against nerve agents in circulation (Billecke et al., 2000; Amitai et al., 2006; Fig. 52.2). Levels and genetic variability of PON1 influence sensitivity to these specific substrates caused by Glu/Arg point mutation at position 192 of the human wild-type enzyme (Billecke et al., 2000; Dragonov and Du, 2004). The Glu^{192} mutant is about three times more active than the Arg^{192} variant (Dragonov and Du, 2004). Despite these differentiations, rather low catalytic activity of recombinant human PON1 expressed in HEK cells occurred as a moderate stereoselective process characterized by preferred cleavage of the less toxic P(+)C(+)-enantiomer of soman (k_{cat} 1030 min^{-1}), which happens twice as fast as for the other three stereoisomers (Yeung et al., 2007, 2008; see Table 52.3). Nordgren et al. (1984) used an enzyme isolated from swine kidney, which they called "phosphoryl phosphatase," to incubate purified enantiomers of soman in vitro. Whereas the less toxic P(+)-isomers (R_PS_C- and R_PR_C-soman) were hydrolyzed very rapidly ($\tau_{1/2} = 2$ min under experimental conditions), both highly toxic P(−) forms (S_PS_C- and S_PR_C-soman) showed much higher stability ($\tau_{1/2}$ 60−120 min). It appears likely that the predominant enzyme isolated was PON1. No stereoselective effects in the catalytic PON1 mediated hydrolysis of cyclosarin and soman using recombinant mammalian material from *Escheria coli* were detected by Amitai et al. (2006). No (+)-cyclosarin was detected in hemolyzed blood samples taken from swine after i.v. administration of racemic (±)-cyclosarin, whereas the (−)-cyclosarin enantiomer was present for at least 20 min after exposure (Reiter et al., 2007). These authors suppose that this phenomenon was due to rapid enzymatic and nonenzymatic hydrolysis in blood in vivo, but they do not discuss the potential role of PON1 explicitly. Yeung et al. (2007) conclude that variations in catalytic efficiency of hPON1 toward soman enantiomers are due to differing Michaelis−Menten constants (K_M), characterizing the stability of the enzyme−substrate complex. Consequently, site-directed mutagenesis of recombinant enzymes could cause reduction of K_M for the more toxic enantiomers, improving its hydrolyzing capacity. A 10- to 100-fold increase in catalytic activity of wild-type hPON1 is

TABLE 52.3 Catalytic constants for hydrolysis of nerve agents and rate constants of esterase inhibition.

	Inhibition rate constant, k_i (M^{-1} min^{-1})								k_{cat} (min^{-1})
	Bovine	Electric eel	Human	Minipig	Pig	Equine	Human	Rat plasma	hr wt
	AChE	AchE	AChE	AChE	AChE	BChE	BChE	CarbE	PON1
Soman									
C(±)P(±)-soman	5×10^7 [a]	1.5×10^8 [a]	9.2×10^7 [b,c]			1.29×10^7 [d]	2.8×10^8 [b]	0.51×10^7 [d]	7,500 [e,f]
C(+)P(+)-soman	$<1 \times 10^4$ [a]	$<5 \times 10^3$ [a]	2×10^3 [g,h]			1.7×10^6 [g]	6×10^6 [g]		$1{,}030 \pm 94$ [i]
C(−)P(+)-soman	$<1 \times 10^4$ [a]	$<5 \times 10^3$ [a]	2×10^3 [g,h]			1.2×10^5 [g]			593 ± 54 [i]
C(+)P(−)-soman	1.75×10^8 [a]	2.8×10^8 [a]	8×10^7 [g,h]			1×10^7 [g]	5×10^6 [g]	3×10^7 [j]	553 ± 163 [i]
C(−)P(−)-soman	2.7×10^7 [a]	1.8×10^8 [a]	1.5×10^8 [g,h]			6×10^7 [g]	4×10^7 [g]	1×10^6 [j]	501 ± 45 [i]
Sarin									
(±)-sarin	1.51×10^7 [d]		3.2×10^7 [b,c]			0.56×10^7 [d]	3.2×10^7 [b]	0.30×10^7 [d]	
(+)-sarin	$<3 \times 10^3$ [k]								
(−)-sarin	1.4×10^7 [k]								
Cyclosarin									
(±)-cyclosarin			4.21×10^8 [l,c]	4.84×10^8 [l]	4.84×10^8 [l]		7.2×10^8 [b]		25,400 [e,f]
VX									
(±)-VX	3.23×10^7 [d]		9.91×10^7 [l,c]	5.61×10^7 [l]	4.43×10^7 [l]	6.3×10^7 [d]		1.51×10^3 [d]	
(+)-VX	2.0×10^6 [k]								
(−)-VX	4.0×10^8 [k]								
VR									
(±)-VR			4.60×10^8 [l,c]	1.95×10^8 [l]	1.88×10^8 [l]				
CVX									
(±)-CVX			3.06×10^8 [m,c]		1.46×10^8 [m]				

Rate constants for inhibition of most prominent serine esterases by nerve agents differ significantly depending on the nature of enzyme, its originating species, and stereoisomers of the agent. It appears obvious that for the depicted G agents and VX, the P(−)-isomers are much more effective inhibitors than their corresponding P(+)-forms. In contrast, hydrolysis of the latter isomers happens faster than that of the toxic P(−)-variants. Hydrolytic stability is determined by PON1 activity characterized by its catalytic constant k_{cat} (last column). Both properties (inhibition and hydrolysis) cause higher toxicity for P(−)-agents in vivo selectively (Table 52.4). AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CarbE, carboxylesterase; CVX, Chinese VX; hr wt, human recombinant wild type; k_{cat}, catalytic constant for hydrolysis; PON1, paraoxonase 1; VR, Russian VX.

[a] *Benschop et al. (1984)*.
[b] *Bartling et al. (2007)*.
[c] *Human RBC AChE.*
[d] *Maxwell and Brecht (2001)*.
[e] *Amitai et al. (2006)*.
[f] *Recombinant rabbit PON1 from* E. coli.
[g] *Ordentlich et al. (1999)*.
[h] *Recombinant human AChE.*
[i] *Yeung et al. (2007)*.
[j] *Sweeney et al. (2006)*.
[k] *Benschop and de Jong (2001)*.
[l] *Worek et al. (2008)*.
[m] *Aurbek et al. (2006)*.

expected to allow effective protection against incorporated nerve agents (Amitai et al., 2006; Rochu et al., 2007; Masson and Rochu, 2009). Masson and Rochu provided Chapter 70, Pyridinium oximes in the treatment of poisoning with organophosphorus compounds, of the previous edition. Following a directed evolution process, Goldsmith et al. (2012) succeeded in producing a recombinant variant with more than 340-fold increased catalytic activity. In addition, stereoselectivity was reversed to preferred hydrolysis of the more toxic $S_P(-)$-isomer of cyclosarin. Recently, the aforementioned PON1 mutant was successfully tested against $2*LD_{50}$ cyclosarin in a guinea pig model. Prophylactic administration of the enzyme prevented death, as well as signs and symptoms of GF poisoning (Worek et al., 2014a). In addition, this enzyme showed a catalytic activity against a broad spectrum of alkyl methylfluorophosphonates. Engineering efficient recombinant human PON1 is a current challenge in medical defense research, intending to yield an effective and biocompatible therapeutic applicable for a wide range of nerve agents exhibiting sufficient activity toward all relevant isomers (Amitai et al., 2006; Valiyaveettil et al., 2011; Kirby et al., 2013). Fig. 52.2 displays its role in biotransformation and elimination.

- Senescence Marker Protein-30 (SMP30): The human senescence marker protein-30 (SMP30 or regucalcin, primary Swiss-Prot accession No. Q15493) is expressed by hepatocytes and plays a role in regulation of plasma membrane Ca^{2+}-pumping activity, with the potential to rescue cells from high calcium level-induced apoptosis (Kondo et al., 2004). The expression of this liver enzyme decreases with aging. SMP30 was originally identified in rat liver and exhibits a 65% amino acid similarity to PON1 in rat species, but does not show catalytic PON1 activity toward the hydrolysis of paraoxon (Billecke et al., 1999). In contrast, mouse and rat SMP30 hydrolyze diisopropylfluorophosphate (DFP), an OPC related to nerve agents (Kondo et al., 2004). It may be speculated that this enzyme is also involved in nerve agent biotransformation in liver. This assumption is supported by the findings of Little et al. (1989), who observed hydrolysis of sarin, soman, and tabun by an enzyme derived from rat liver homogenate. Recombinant human material might also represent a valuable countermeasure for nerve agents and was expressed in *E. coli* as a properly folded and active protein (Choi et al., 2010).
- Prolidase: Prolidase is a Mn^{2+}-dependent enzyme of 54 kDa that was purified from fibroblast cells, erythrocytes, kidney, and liver (EC 3.4.13.9) and exhibits a primary catalytic activity toward peptide-bond cleavage of dipeptides containing a C-terminal proline or hydroxyproline residue. In addition, it was found to hydrolyze G-type nerve agents tabun, sarin, soman, and cyclosarin by liberating the respective leaving groups in vitro with K_M values in the millimolar range. Unfortunately, recombinant human material expressed in *E. coli* exhibited a stereoselective preference for less toxic S_P-isomers; thus, it may not degrade the poison efficiently (diTargiani et al., 2010). This enzyme will affect the toxicokinetics of nerve agents and may represent an additional bioscavenger candidate when activity can be enhanced and stereoselectivity reversed.
- Diisopropyl Fluorophosphatase (DFPase): Some enzymes were isolated, but not unambiguously identified, from swine kidney, which exhibit DFP-cleaving activity and were denominated diisopropyl fluorophosphatase (DFPase) in older swine (Nordgren et al., 1984). This DFPase belongs to the class of A-esterases acting on DFP, tabun, and organofluorophosphates (e.g., cyclosarin, sarin, and soman). Nowadays, most recent studies define DFPase (EC 3.1.8.2, formerly assigned as EC 3.8.2.1) as a calcium-dependent PTE identified in squid *Loligo vulgaris* exhibiting unique structural properties (Blum et al., 2006). This squid-type DFPase has not yet been identified in mammalian organisms.

52.3.7.2 Nonmammalian enzymes

Several other enzymes from bacteria [e.g., PTEs or OPH, from *Pseudomonas diminuta* or *Flavobacterium* sp., OP acid anhydrolase (OPAA), from *Alteromonas* sp. and prolidases from *Pyroscoccus furiosus*, or squid (DFPase)] are also known to detoxify nerve agents, and it is of interesting relevance for novel noncorrosive decontamination approaches (Blum and Richardt, 2008; Theriot et al., 2011). So far it does not play a role in the toxicokinetics of OPCs in humans (Amitai et al., 2006; Rochu et al., 2007; Masson and Rochu, 2009) but has shown benefit in animal studies. Wille et al. (2016) reported on the PTE mutant C23AL that was administered [intraosseous (i.o.) and i.v.] to guinea pigs poisoned with VX. Animals challenged with the nerve agent (2 LD_{50}) and subsequently treated with PTE survived despite substantial inhibition of AChE in erythrocytes, brain, and diaphragm (Wille et al., 2016). In contrast, promising studies were performed in rodents demonstrating a protective effect of OPH (Wales and Reeves, 2012). However, potential application as detoxifying antidotes remains a future challenge (Ashani et al., 2016; Goldsmith et al., 2016). Promising new strategic approaches on systematic testing of enzyme mutants were presented by Bigley et al. (2019) documenting effective improvement of catalytic activity toward V-type agent hydrolysis. Current review articles on this aspect are provided by Goldsmith and Ashani (2018) and Masson and Lushchekina (2016).

52.3.8 Nonproteinaceous scavengers and hydrolyzing compounds

Scavenging and hydrolysis of OP nerve agents in vivo is a major challenge in therapeutic treatment of poisoning. Compounds that enable degradation of poison to nontoxic products in the body help to minimize the toxic impact of nerve agents. In contrast to bioscavengers like PON1 or BChE, small molecules are much less immunogenic and might be of higher stability and longer half-life, representing more favorable properties for antidotal treatment. Following this idea, structures of modified β-cyclodextrins (β-CD) are currently optimized for improved hydrolysis of G- and V-type nerve agents (Estour et al., 2013). In vitro studies indicate reversible binding to β-CD as noninclusion (interaction with the outer surface) and as inclusion complexes (interaction inside the hydrophobic cavity) initiating hydrolysis that might occur with enantioselective preference (Müller et al., 2013). Recently, a β-cyclodextrin derivative bearing a pyridinium oximate in 6-position of one glucose unit was synthesized and shown to possess a promising detoxification potential against a variety of alkyl methylfluorophosphonates in vitro (Bierwisch et al., 2014; Kranawetvogl et al., 2015). Recent studies showed that the nerve agent-degrading effect of glucose functionalized with oximate and hydroxamic acid is a result of stoichiometric binding but not of a catalytic process (Bierwisch et al., 2016). Prophylactic i.v. injection of the β-cyclodextrin derivative prevented systemic toxicity in cyclosarin ($\sim 2LD_{50}$) poisoned guinea pigs and preserved brain AChE activity (Worek et al., 2014b). Such compounds will seriously affect toxicokinetic behavior of poisons and support the elimination process. In addition, derivatives of calixarenes were also tested but need much more improvement of efficacy (Schneider et al., 2016; Ede et al., 2018).

52.3.9 Formation of protein adducts

Besides direct enzymatic hydrolysis of nerve agent substrates, numerous additional proteins are present in organisms that allow covalent binding to OPCs, contributing to detoxification of the poison load (Fig. 52.2). Serine esterases are especially predominant targets of nerve agents mostly undergoing irreversible adduct formation. Nevertheless, more than 75% of serine hydrolases (B-esterases) present in plasma and tissues are essentially unknown with respect to their interaction with OPCs (Casida and Quistad, 2005).

52.3.9.1 Carboxylesterase

Ubiquitous glycosylated carboxylesterases (CarbE, EC 3.1.1.1), formerly named *ali-esterases*, are B-esterases belonging to the multigene enzyme superfamily of α/β hydrolases (Hosokawaand Satoh, 2006; Satoh and Hosokawa, 2006). In principle, this class of isozymes plays a major role in pharmacokinetics by hydrolytic biotransformation of exogenous ester-drugs and ester-prodrugs. However, their physiological function remains unclear (Satoh and Hosokawa, 2006).

Carboxylesterases are very important serine esterases in plasma of nonhuman species that bind nerve agents with broad specificity, representing the major determinant for in vivo detoxification, especially in mice and rats (Maxwell and Brecht, 1991; Fig. 52.2). Apart from catalytic CarbE activity in plasma, it is also found in several tissues and organs, including the brain, lung, kidney, and liver, thus indicating that poison decrease was not only in the circulation (Satoh et al., 2002). Microsomal liver carboxylesterase 1, which is also referred to as *egasyn*, is loosely associated with a β-glucuronidase (BG) complex (Fujikawa et al., 2005). Organophosphate-inhibited egasyn causes cleavage of this complex, releasing elevated quantities of BG into circulation. Thus, BG plasma activity may serve as a sensitive biomarker for OP poisoning (Inayat-Hussain et al., 2007).

Interaction of CarbE with nerve agents follows a kinetic of first order characterized by inhibition of CarbE at the active site serine residue described by a bimolecular rate constant, k_i (Maxwell and Brecht, 2001). For noncharged nerve agents (e.g., sarin and soman), the k_i of rat serum CarbE was found to be greater than $10^6\ M^{-1}\ min^{-1}$, whereas cationic substrates (e.g., VX) are converted with poor reactivity ($k_i < 10^4\ M^{-1}\ min^{-1}$). This specificity is explained by the electrostatic characteristics of the large active site containing only a few cation-Π bonding and anionic residues (Maxwell and Brecht, 2001; Satoh and Hosokawa, 2006).

Covalent binding of OPCs to CarbE is considered an irreversible reaction of 1:1 stoichiometry, resulting in adducts that do not age (Maxwell and Brecht, 2001). In contrast, spontaneous pH-dependent reactivation liberates the enzyme, making it accessible for additional detoxification (though only to a very limited extent). At physiological pH in vitro, spontaneous reactivation (specified by the rate constant k_r) is a poison-dependent process showing significantly different velocities depending on the size of the inhibitor (steric demand). Whereas reactivation of VX- and sarin-inhibited rat serum CarbE was faster (k_r $4.2 \times 10^{-3}\ min^{-1}$ and $3.8 \times 10^{-3}\ min^{-1}$), reactivation for soman and VR was about 10 times slower (k_r $0.44 \times 10^{-3}\ min^{-1}$ and $0.52 \times 10^{-3}\ min^{-1}$; Maxwell and Brecht, 2001). Meanwhile, Hemmert et al. (2011) produced a recombinant variant of human CarbE1 that spontaneously dephosphorylates 33,000-fold faster than the wild type after inhibition with sarin, soman, and cyclosarin. This effect was achieved by implementing a pair of histidine

and glutamic acid residues proximal to the catalytic triad without changing the high-affinity binding. In addition to these differences, CarbE exhibits stereoselective properties. Nordgren et al. (1984) demonstrated that CaE from swine liver binds to the less toxic P(+)C(+)-enantiomer of soman (R_CR_P) most efficiently, whereas conversion of highly toxic P(−)C(+)-soman (R_CS_P) happens much more slowly (Table 52.3). This fact indicates that degradation of incorporated nerve agents is primarily targeted to less toxic enantiomers, minimizing the protective effect of CaE. In contrast to G agents, no prominent enantiomeric selectivity was evident for sequestration of VX after p.c. administration (Van der Schans et al., 2003).

Consequently, striking species-dependent differences in LD_{50} values of OPCs are mainly due to variable concentrations of endogenous CarbE acting as a bioscavenger in blood (Tables 52.2 and 52.3). Whereas mice and rats exhibit high CarbE activities, activity in rabbits and guinea pigs appears moderate. In contrast, dogs and primates, as well as humans, possess only little or no CarbE (Benschop and de Jong, 2001). These relations are obvious from s.c. soman LD_{50} values obtained from mice and guinea pigs after inhibition of CarbE (10.2 and 12.2 μg/kg), which were very similar to those obtained for dogs and primates without inhibition (9.1 and 13.0 μg/kg; Maxwell and Brecht, 1991). In contrast, the corresponding soman s.c. LD_{50} values for untreated CarbE are much higher for mice (113 μg/kg) and guinea pigs (28.2 μg/kg; Maxwell and Brecht, 1991). For these reasons, guinea pigs are most often used for in vivo toxicity and toxicokinetic studies intending to examine a small laboratory animal model predictive for humans. Missing CarbE activity in humans hinders efficient detoxification in vivo, thus keeping the toxic effect of nerve agents (Due et al., 1993). Consequently, the value of CarbE as a bioscavenger for effective clearance of OP poison from the circulation has led to therapeutic concepts that were applied successfully to rodents and nonhuman primates (Maxwell and Brecht, 2001). Feasibility for human organisms has yet to be demonstrated.

52.3.9.2 Acetyl monoalkylglycerol ether hydrolase

KIAA1363 (primary Swiss-Prot accession No. Q6PIU2) is a human serine hydrolase derived from acetyl monoalkylglycerol ether hydrolase (AcMAGE, EC 3.1.1.) present in the brain, lung, heart, and kidney, which is involved in tumor cell invasiveness and lipid metabolism, but can also detoxify OPCs by hydrolysis of the reactive leaving group following transient binding to the active site serine residue (Nomura et al., 2008; Fig. 52.2). This capability has been demonstrated for the pesticide chlorpyrifos and its more toxic oxon derivative. Activity toward nerve agent hydrolysis is expected, but it has not been shown so far.

52.3.9.3 Acetylcholinesterase

AChE (EC 3.1.1.7) from vertebrates is deduced from a single gene (Massoulie, 2002) expressing identical enzyme primary structures on the surface of RBCs, in synapses and different organs. Inhibition of this serine-esterase causes the most prominent pathophysiological effects, determining the severity of poisoning. Covalent binding to AChE can also be considered a step in poison elimination (Fig. 52.2), which reduces the amount of incorporated poison, though only to a small extent.

AChE activity in whole blood differs significantly among mammalian species, which makes it relevant for toxicokinetic consideration. Humans exhibit a very high AChE activity in blood (651 mU/μmol hemoglobin, Hb) in contrast to smaller values for miniature pigs (297 mU/μmol Hb) and pigs (190 mU/μmol Hb; Worek et al., 2008). The bimolecular rate constants (k_i) for nerve agent-induced inhibition of AChE vary among different species. Table 52.3 gives an overview of corresponding inhibition kinetics. The k_i for inhibition of human AChE by VX, for example, is about twice as high as for pig AChE (Aurbek et al., 2006). These differences must be considered when assessing interspecies toxicokinetic data.

Nerve agents show enantioselective inhibition kinetics when reacting with AChE. Chirality at the central phosphorus atom plays the predominant role, affecting higher rates of inhibition for the P(−)-isomers (S_P) than for the P(+)-forms (RP; Nordgren et al., 1984). Benschop and de Jong (2001) made great efforts to look at this stereoselectivity topic, and they determined the corresponding inhibition rate constants (k_i) for electric eel AChE in vitro (Table 52.3). Both P(−)-diastereomers of soman (S_PS_C- and S_PR_C-soman) exhibit very high k_i values ranging from $1.8-2.8 \times 10^8\ M^{-1}\ min^{-1}$, which documents their high toxicity. In contrast, the less toxic P(+)-diastereomers (R_PR_C- and R_PS_C-soman) are characterized by k_i values that are 100,000 times smaller ($<5 \times 10^3\ M^{-1}\ min^{-1}$; Benschop and de Jong, 2001). Similar results were obtained for sarin enantiomers, which differ by a factor of 5000, revealing a k_i of $1.4 \times 10^7\ M^{-1}\ min^{-1}$ for the S_P-(−)-form (Table 52.3). VX enantiomers exhibit the smallest differences, because the k_i of the more toxic S_P-(−)-VX ($4 \times 10^8\ M^{-1}\ min^{-1}$) is 200 times higher than for the RP-(+)-enantiomer (Table 52.3; Benschop and de Jong, 2001). These k_i data correspond to the order of experimentally determined LD_{50} values in mice, not considering the impact of hydrolyzing enzymes in vivo which also exhibit stereoselective kinetics for hydrolysis (Table 52.4; Benschop and de Jong, 2001).

TABLE 52.4 Acute lethality in mice caused by stereoisomers of OP nerve agents.

Nerve agent	LD_{50} (μg/kg)
Stereoisomers	**Mouse**
Soman	
C(±)P(±)-soman	156 (s.c.)[a]
C(+)P(+)-soman	<5000 (s.c.)[b]
C(−)P(+)-soman	<5000 (s.c.)[b]
C(+)P(−)-soman	99 (s.c.)[a]
C(−)P(−)-soman	38 (s.c.)[a]
Sarin	
(±)-sarin	83 (i.v.)[b]
(+)-sarin	
(−)-sarin	41 (i.v.)[b]
VX	
(±)-VX	20.1 (i.v.)[b]
(+)-VX	165 (i.v.)[b]
(−)-VX	12.6 (i.v.)[b]

Higher rate constants of P(−)-isomers for inhibition of AChE in combination with their minor susceptibility to enzymatic hydrolysis by mammalian PTEs cause eminently different lethal doses in vivo.
[a]*Benschop et al. (1984)*.
[b]*Benschop and de Jong (2001)*.

AChE on the surface of RBCs shows a turnover rate of about 1% per day, enabling complete exchange within approximately 100 days after exposure (Sidell and Borak, 1992). In contrast, regeneration of synaptic AChE is assumed to be faster, reaching 7%−10% turnover rates, an estimation determined by animal experiments (Grubić et al., 1981; Brank et al., 1998; Eddleston et al., 2008b). Both are of importance for therapeutic monitoring of poisoning, as well as for verification of exposure following bioanalytical methods.

- Spontaneous Reactivation of AChE: Although inhibition of AChE is mainly discussed as an irreversible reaction, detailed kinetic investigations consider the process of spontaneous reactivation re-releasing an active enzyme and a hydrolyzed detoxified agent (Fig. 52.2). Rate constants for spontaneous reactivation (k_s) of human AChE adducts are very small, as measured for some sarin analogs (0.01−0.052 h^{-1}), whereas the k_s for sarin, cyclosarin, and VX was too small to provoke an experimentally detectable effect (Bartling et al., 2007). Sidell and Groff (1974) demonstrated that spontaneous reactivation of RBC AChE inhibited by VX happens much faster than for sarin, which is in accordance with recent human k_s data on V agents VX 0.021 h^{-1}, VR 0.039 h^{-1}, and Chinese VX (CVX) 0.171 h^{-1} (Aurbek et al., 2006). Following point mutations in recombinant human AChE spontaneous reactivation rates for VX, VR, and CVX could be increased up to 110-fold (Trovaslet-Leroy et al., 2011). However, the toxicokinetic relevance of spontaneous reactivation is obvious from the liberation of free esterase accessible for reinhibition with 1:1 stoichiometry.
- Aging of AChE: The cleavage of an organic substituent from the phosphorus atom, which is bound to the side chain of an amino acid residue from a protein, is described by the aging process (Fig. 52.2). Promoted by specific ionic interactions of neighboring amino acid residues in AChE (and BChE), the remaining P-OH function will undergo deprotonation, leaving a negatively charged phosphorus moiety (Fig. 52.2). Chandar and Ganguly (2013) investigated the aging process of soman-inhibited AChE in silico by density functional theory (DFT) and found that dealkylation of the pinacolyl-group and migration of the methyl-group take place simultaneously, catalyzed by histidine 440 and tryptophan 84 residues located in the catalytic triad and catalytic anionic subsite, respectively. Whereas esterase adducts of sarin and soman age by cleaving alkoxy moieties from the central phosphorus atom, tabun loses its dimethylamine moiety (P−N scission) instead of its ethoxy group (Elhanany et al., 2001). These aged enzyme adducts are not accessible for reactivation by common antidotal oximes, limiting the success of therapeutic treatment of poisoning with OPCs. The soman−AChE adduct is known for very rapid aging ($\tau_{1/2} \approx 2$ min), causing severe problems in clinical treatment (Worek et al., 2005). Aging half-lives of phosphylated AChE and phosphylated BChE depend on the nature of the inhibiting OPC. Whereas GF-inhibited AChE exhibits a half-life of 8.7 h, the corresponding BChE derivative ages much more rapidly (2.2 h; Worek et al., 1998). The order of $\tau_{1/2}$ for aging of human AChE−nerve agent adducts was found to be, in ascending order, soman (2 min), sarin (3 h), cyclosarin (7 h), tabun (19 h), and VX (36.5 h; Worek et al., 2005). V agent-inhibited RBC-AChE ages very slowly in humans, allowing therapeutic intervention with oximes for a longer period lasting several days (Sidell and Groff, 1974; Thiermann et al., 2007).

52.3.9.4 Butyrylcholinesterase

BChE (EC 3.1.1.8), formerly named *pseudocholinesterase*, is synthesized in the liver and present in blood (5 μg/mL), the synapse of neuromuscular junctions, and glia

cells and axons of white matter in the brain in numerous allelic variants (Massoulie, 2002). Although BChE has long been considered a nonfunctional vestigial analog of AChE, recent findings point out a possibly more prominent role; especially in mice, where the total amount of BChE in the body is 10 times as high as AChE (Duysen et al., 2001). Correspondingly, it was observed that the activity of BChE in human whole blood was significantly higher than that of AChE (Worek et al., 2008). This led to speculation that BChE may play a backup role for insufficient AChE activity in neurotransmission as deduced from the physiology of AChE knockout mice (Duysen et al., 2001) and may serve as a safeguard against diffusion of ACh into the bloodstream (Massoulie, 2002). However, mandatory experimental evidence is still missing.

Highly glycosylated BChE is a prominent target of OPCs, acting as a protective biological stoichiometric scavenger averting damage to neuronal AChE (Kolarich et al., 2008). However, common nerve agents may exhibit significantly differing inhibition rate constants (k_i); for AChE and BChE, they are approximately in the range of 10^7–10^9 M^{-1} min^{-1} (Bartling et al., 2007). Table 52.3 shows a comparison of the inhibitory potency of nerve agents against AChE and BChE. In vivo studies in humans suggest that VX preferentially inhibits RBC AChE much more effectively than BChE, resulting in 70% and 20% inhibition, respectively (Sidell and Groff, 1974). In addition to agent-dependent k_i values, there is a striking stereoselective dependency in BChE inhibition. The more toxic soman P(−)-enantiomers (S_P) inhibit with preference when compared to their corresponding P(+)-forms (RP; Table 52.3; Nordgren et al., 1984). However, differences are not as pronounced as for AChE, but point out effective detoxification properties of endogenous BChE.

Interestingly, no clinical features result from inhibited BChE in vivo (Eddleston et al., 2008b). The status of plasma BChE activity is, despite all concerns, a commonly recommended measure to monitor the progress of chemical injury (Eddleston et al., 2008a).

In contrast to all studies on VX toxicokinetics and toxicodynamics published so far, Dorandeu et al. (2008) reported an unexpected and not-yet-clarified phenomenon that emerged after i.v. administration of VX to isoflurane-anesthetized and ventilated swine without oxime therapy. Time-resolved measurement of esterase activities during the experimental period of poison application revealed very fast rebound of BChE activity (from 70% inhibition to 30% within 1 h), while retaining nearly complete inhibition of whole-blood cholinesterase. Design and control experiments allowed the following explanations to be excluded: (1) spontaneous reactivation, which should happen much more slowly; (2) induced hypoalbuminemia liberating albumin as scavenger competing with BChE; and (3) stimulated release of hydrolyzing enzymes like PON1. More probably, this observation is explained by an increased biosynthesis of BChE in the liver, an elevated release of BChE from other organs (e.g., heart, lung, or pancreas), or both. Future studies will possibly shed more light on this curious and interesting phenomenon.

As total replacement of BChE by synthesis in the liver happens within a couple of weeks, this rather long period allows experimental verification of OP poisoning, even when blood samples from poisoned humans are collected with significant delay to the time of exposure (Sidell and Borak, 1992). Therefore, detection of BChE adducts by means of modern mass spectrometric methods is the state-of-the-art technique for proving exposure to OP nerve agents (Carol-Visser et al., 2008; Noort et al., 2006; John et al., 2008). Apart from that specific serine residue that is phosphylated by organophosphorus pesticides and nerve agents, Schopfer and Lockridge (2019) identified additional amino acids being phosphylated after in vitro incubation of BChE with clorpyrifos oxon including 10 tyrosine, 14 lysine, two histidine, and two threonine residues. Whether these modifications are of relevance for the enzymatic activity remains unclear.

The relevant enzyme adducts at the serine residue of the esteratic center may undergo the previously described consecutive reactions: spontaneous reactivation and aging. Jiang et al. (2013) demonstrated that human BChE can be inhibited by both enantiomers of tabun, resulting in stereospecific aging processes showing deamination for S_P-tabun and O-dealkylation for R_P-tabun. In general, aging of BChE nerve agent adducts occurs with highly different agent-dependent periods of half-change ($\tau_{1/2}$) as determined in vitro by Worek et al. (2005). Whereas the soman adduct typically exhibits by far the shortest $\tau_{1/2}$ (less than 1 min), cyclosarin appears much more stable ($\tau_{1/2}$ 2.2 h), followed by tabun ($\tau_{1/2}$ 7 h) and sarin ($\tau_{1/2}$ 12 h). The adduct of VX was the most stable BChE derivative characterized by a $\tau_{1/2}$ of 77 h. Spontaneous reactivation by simple hydrolysis of the serine−phosphorus bond was observed for cyclosarin, with a half-life of 20 h and much longer times for VX (63 h) and sarin (63 h; Worek et al., 2005). These data demonstrate that inhibition of wild-type BChE is nearly irreversible, lowering the amount of incorporated toxic OPCs significantly in a stoichiometric manner. Therefore, protection against nerve agent doses of up to $5.5 \times LD_{50}$ (soman and VX) was achieved with exogenous BChE from different species applied prophylactically i.m. to the guinea pig, rhesus monkey, and cynomolgus (Lenz et al., 2007). It is supposed that a dose of 200 mg BChE/70 kg will be sufficiently protective in humans against $2 \times LD_{50}$ of soman (Saxena et al., 2008). Based on this valuable protective capacity, current efforts are under investigation to use BChE from recombinant

(e.g., transgenic plants or milk of transgenic animals) or natural sources for therapeutic/prophylactic treatment of nerve agent poisoning (Chilukuri et al., 2005; Lockridge et al., 2005; Huang et al., 2007; Lenz et al., 2007, 2010; Geyer et al., 2010; Saxena et al., 2011; Mumford et al., 2013). The plasma half-life of recombinant preparations is significantly prolonged by fusion to albumin (Huang et al., 2008) or pegylation (attachment of polyethyleneglycol; Chilukuri et al., 2005; Sun et al., 2013), or by polysialylation (Ilyushin et al., 2013). In addition, enhanced rapid spontaneous reactivation will deblock the enzyme, thus being accessible for subsequent binding to another OP molecule. This strategy is followed by site-directed mutagenesis of BChE, leading to a 10^5-fold increase in dephosphorylating activity (Casida and Quistad, 2005). Accordingly, substitution of histidine 117 by glycine in engineered human BChE generated a significant amount of OP hydrolase activity (Nachon et al., 2011). Feasibility for the human body still has to be shown.

52.3.9.5 Albumin

Although albumin is present in blood in high concentrations (0.6 mM; 41 g/L; 50%−60% of total plasma protein), it does not represent an effective scavenger for nerve agent detoxification in vivo, hampered by its slow reaction velocity (Li et al., 2008). Nevertheless, sarin and soman bind to albumin at active site tyrosine residue 411, as shown in neat buffered solution and crude human plasma. This inhibits the enzymatic acylamidase and esterase activity of albumin (Black et al., 1999; Li et al., 2007, 2008; Fig. 52.2). Besides tyrosine 411, additional amino acid residues capable of phosphorylation by nerve agents and pesticides were described by John et al. (2010) after matrix-assisted laser desorption/ionization (MALDI)−mass spectrometry (MS) as well as by Schopfer and Lockridge (2019) and Fu et al. (2019) using liquid chromatography-tandem mass spectrometry (LC MS/MS) analysis. Albumin also exhibits slight hydrolyzing catalytic activity against OPCs (Vilanova and Sogorb, 1999). Concerning enantio-selectivity in phosphorylation of albumin by soman, contradictory results have been presented in recent studies. Li et al. (2008) did not find any enantiomeric preference, and Yeung et al. (2008) demonstrated that human serum albumin preferentially binds to the less toxic C(±)P(+)-enantiomer. However, the albumin adduct is very stable in terms of spontaneous reactivation, exhibiting extended periods of half-life (6.5 days at 25°C, pH 8.0 and 20 days at 22°C, pH 7.4); in addition, it does not show any aging phenomena (Li et al., 2007, 2008; John et al., 2010). Derivatized albumin serves as a biomarker for nerve agent exposure detectable by modern mass spectrometric techniques (Black et al., 1999; Peeples et al., 2005; Li et al., 2007, 2008; Williams et al., 2007). Recently, a novel class of disulfide adducts was presented that is produced from albumin in the presence of V-type nerve agents and pesticides containing a thiol-leaving group (John et al., 2018a,b; Kranawetvogl et al., 2016, 2017, 2018a,b). The thiol-containing leaving group undergoes disulfide formation with the Cys^{34} residue of albumin which—in the native structure—is the only free, nondisulfide bridged cysteine residue within that protein. Whether these chemical modifications effect any changes in the 3D structure or functionality of albumin remains unclear, but the derivatized Cys^{34} residue can be used for bioanalytical detection and thus for verification of poisoning. The adducted albumin is proteolyzed by pronase producing the dipeptide Cys^{34}Pro that is modified at the cysteine side chain containing the disulfide-linked leaving group (John et al., 2018a,b; Kranawetvogl et al., 2016, 2017, 2018a,b).

52.3.9.6 Keratins

Keratins are the most abundant proteins in the stratum corneum, exhibiting molecular weights of about 50−100 kDa. Formation of intermediate filaments assembled from bundles of keratin monomers cause durability and insolubility in water, representing the protective barrier of the body. Verstappen et al. (2012) found that VX and OP pesticides covalently bind to tyrosine residues in human keratins when exposed in vitro. MS/MS analysis revealed phosphorylation in keratin 1, 6, and 10, results that are very similar to a report from Schopfer et al. (2010) documenting phosphorylation of tyrosine induced by in vitro incubation of chlorpyrifos-oxon with human keratin 1, 2, 9, and 10. No distinct binding motifs or consensus sequences have been identified so far that explain preferred phosphorylation at specific tyrosine residues (Schopfer et al., 2010). In addition, adducts were also found at lysine residues in keratin 1 and 10. Corresponding phosphoamidate bonds showed unexpected stability (Grigoryan et al., 2009). Such binding processes might contribute to general skin absorption phenomena as observed after cutaneous exposure to VX (Chilcott et al., 2005). No pathophysiological consequences are obvious at present. These phosphorylation reactions minimize the total amount of toxic nerve agents penetrating into circulation, representing a kind of natural scavenging barrier.

52.3.9.7 Ubiquitin

Ubiquitin is a small endogenous and ubiquitous protein (approximately 8600 Da) with a nearly globular confirmation (Welchman et al., 2005). In vivo, it appears either as free (extracellular space, for example, plasma, urine, cerebrospinal fluid) or anchored molecules covalently bound to any target protein (intracellular space). Such conjugates often contain multiple ubiquitin molecules connected to

long chains (poly-ubiquitinylation). The kind and extent of ubiquitin chains are crucial parameters that determine the fate of the protein with respect to proteasomal degradation. Chain prolongation requires one of seven free lysine residues to react with the C-terminal glycine of another ubiquitin molecule. Therefore, the presence of free, chemically unaltered ε-amino side chains is essential for correct biological function.

Recently, adducts of ubiquitin produced in vitro with VX, VR, and CVX were identified by different mass spectrometric methods, documenting that phosphorylation untypically occurred in at least six of the seven lysine residues selectively (Schmidt et al., 2014). An additional adduct at a tyrosine residue was also found, but to a much lesser extent. Intramolecular cyclization between the derivatized lysine and adjacent glutamic acid residues was detected. Such a reaction has never been described before for any other phosphylated protein but has been shown meanwhile for additional proteins including aprotinin, casein alpha S1, human serum albumin, BChE and tubulin alpha 1A (Schopfer and Lockridge, 2019). Cyclization will dramatically change the tertiary structure of ubiquitin and presumably of other proteins. All these chemical and structural changes after reaction with V agents will surely affect biological function. It is not clear whether phosphylated ubiquitin is produced in in vivo poisoning scenarios or whether it is relevant to any pathophysiological situation.

52.3.9.8 Additional proteins

By means of modern, highly selective, and high-resolving mass spectrometry coupled to effective chromatographic separation, a number of mammalian proteins were shown to form covalent adducts in vitro. These targets include, for example, α_2-glycoprotein 1, human transferrin, kinesin 3C motor domain 5, bovine tubulin α and β, actin α skeletal muscle, chymotrypsinogen, mouse transferrin, ATP synthase, adenine nucleotide translocase I, aprotinin, casein alpha S1, and porcine pepsin (Grigoryan et al., 2009; Schopfer et al., 2010; Schopfer and Lockridge, 2019). Nerve agents were found to phosphorylate tyrosine, serine, threonine, and lysine residues predominantly. However, no correlation to any specific disease or evidence for any pathophysiological consequence has been reported. Nevertheless, numerous signs and symptoms in OP poisoning suggest that AChE inhibition is not the only reason behind the entire clinical picture.

Besides diverse nucleophilic side chains of amino acids from a number of endogenous proteins, OP nerve agents also form adducts with other small, nucleophilic molecules relevant for toxicokinetic analysis. Gäb et al. (2010, 2011) demonstrated that sarin, soman, and cyclosarin react with phosphate anions. Such ions are present in vivo or in typical phosphate buffers used for dilution of biological fluids or as solvents for kinetic measurements. G-type nerve agents (Gäb et al., 2011) and VX (Creasy et al., 2012) produce very stable pyrophosphate-like adducts fast enough to compete with sample hydrolysis in aqueous media. Similar reactivity was found for amino alcohol buffers like tris(hydroxymethyl)aminomethane (TRIS), and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), generating *O*-bound esters of methyl-*O*-alkyl phosphonates derived from sarin, soman, and cyclosarin (Gäb et al., 2010). These reactions might be relevant when planning and performing any kind of quantitative and kinetic analysis of G-type nerve agents.

52.3.10 Muscarinic receptors

It has been shown that tabun, sarin, soman, and VX bind to the muscarinic receptor subtype m_2, leading to the assumption that the ACh binding site is deactivated, causing potential additional vulnerability (Fig. 52.2; Silveira et al., 1990). Despite the high affinity of nerve agents, pathophysiological effects seem to be of only minor relevance compared to AChE inhibition. However, binding to receptors will lower the concentration of toxic and reactive OPCs.

52.3.11 Excretion

Sarin and its corresponding nontoxic hydrolysis products (IMPA and additional methylphosphonic acids) are predominantly eliminated via the kidneys, so those organs play a more important role in detoxification than the liver (Little et al., 1986; Waser and Streichenberg, 1988). Urinary excretion happens very rapidly, as demonstrated for single-dose s.c. applications of sarin, cyclosarin, and soman to rats (Shih et al., 1994). The terminal elimination half-life was found to be 3.7 ± 0.1 h for sarin and 9.9 ± 0.8 h for cyclosarin. Soman showed a biphasic elimination, with terminal half-lives of about 18.5 and 3.6 h (Shih et al., 1994). Maximum peak levels of sarin biotransformation products in urine were detected 10−18 h after exposure (Minami et al., 1997) and, after 2 days, hydrolyzed sarin products had been excreted nearly quantitatively (Shih et al., 1994). Even at 5 days, postexposure soman product recovery was only 62% (Shih et al., 1994). Excretion of soman from blood, liver, and kidneys following chemical and enzymatic hydrolysis is considered a first-order elimination process (Sweeney et al., 2006).

52.3.12 Concentration−time profiles of nerve agents in blood after various routes of administration

Extensive toxicokinetic studies in animal models using rats, marmosets, and guinea pigs were performed for sarin

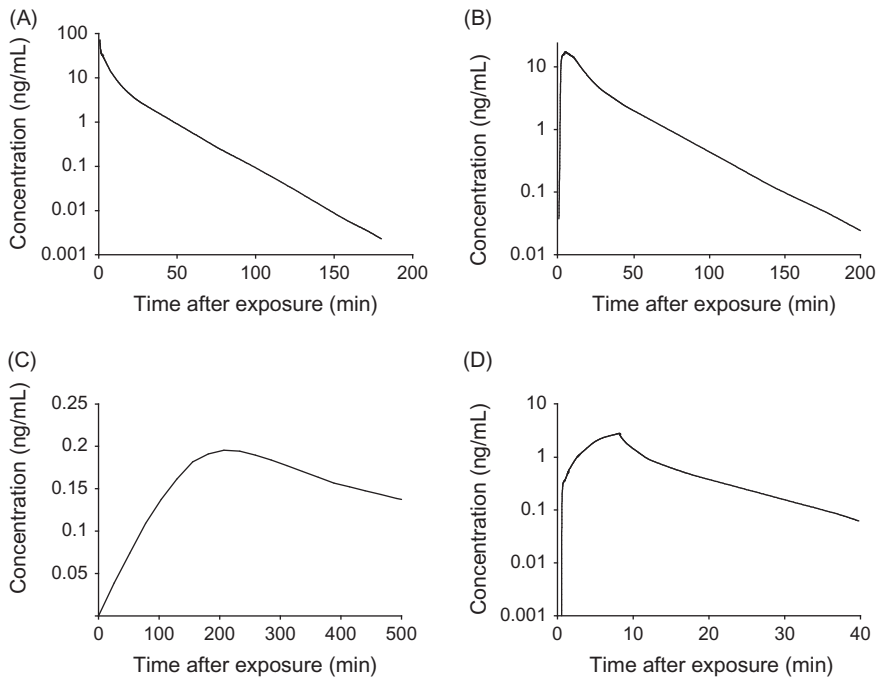

FIGURE 52.3 Concentration−time profiles of highly toxic C(−)P(−) soman and (±) VX in guinea pig blood after administration of nerve agents via different routes of exposure. Guinea pigs were challenged with C(±)P(±)-soman (A, B, D) or (±) − VX (C). (A) Intravenous, $6 \times LD_{50}$ (165 μg/kg); (B) subcutaneous bolus, $6 \times LD_{50}$ (148 μg/kg); (C) percutaneous bolus, $1 \times LD_{50}$ (125 μg/kg); (D) nose-only for soman vapor in air at $0.8 \times LCt50$ (48 mg/m^3 for 8 min). Nerve agents were applied to anesthetized, atropinized, and mechanically ventilated animals. Data fits are according to basic studies presented by Benschop and de Jong (2001; A, B, D) and by Van der Schans et al. (2003; C).

and most often for soman, thereby considering individual concentration−time profiles of different enantiomers (Benschop and de Jong, 2001). The resulting concentration curvatures reflect the combination of toxicokinetic factors of distribution and elimination by hydrolysis and protein binding, as described previously in detail. The next section will briefly summarize the most prominent findings about stereoselectivity and related blood concentration−time profiles. In contrast to many OP pesticides, detailed toxicokinetic data on oral uptake of nerve agents is not available. Therefore, we restrict this section to i.v., s.c., p.c., and respiratory exposure. For more detail, a concise and extensive overview is given by Benschop and de Jong (2001).

52.3.12.1 Intravenous uptake

Direct poison injection into the circulation allows immediate distribution in an organism, but also enables undelayed elimination provoked by plasma components. To illustrate these impacts, we introduce some fundamental results obtained for some G agents, as depicted in Fig. 52.3A (Benschop and de Jong, 2001).

As soon as 0.3 min after i.v. administration of C(±)P(±)-soman to rats, guinea pigs, and marmosets (3−6 LD_{50}), the less toxic C(+)P(+)-enantiomer degrades to nondetectable concentration, whereas its diastereomer C(−)P(+)-soman is detectable for a few minutes longer. This rapid decrease in P(+)-enantiomers is caused by fast enantioselective catalytic hydrolysis (Table 52.3). In contrast, the highly toxic P(−)-diastereomers are detected for up to 1 h or longer, showing a steep initial concentration decrease caused by systemic distribution, protein binding, and hydrolysis, followed by a more moderate concentration decline during the later elimination phase (Fig. 52.3A). CarbE is expected to be the most important scavenger in laboratory animals, being essential for nerve agent elimination and causing high species-dependent variations in LD_{50} values (listed in descending order, rat, guinea pig, and marmoset), which correlate to individual CarbE concentrations (Table 52.2). Resulting concentration curvatures were fitted best by a three-exponential equation (Benschop and de Jong, 2001). Fig. 52.3A gives a representative example of the concentration−time profile of C(−)P(−)-soman in guinea pigs. Corresponding experiments, performed with (±)-sarin applied to guinea pigs, demonstrated that initial distribution of (−)-sarin happened an order of magnitude faster than P(−)-soman, whereas elimination was one order of magnitude slower (Benschop and de Jong, 2001). The reasons for these differences are not clarified yet, but they are expected to be due to different kinetics of hydrolysis and protein binding, causing higher persistence of (−)-sarin.

Similar results of stereospecific kinetics were also observed for tabun after i.v. challenge in swine, showing a shorter terminal half-life elimination for the less toxic (+)-tabun (Tenberken et al., 2010). In accordance, the (−)-cyclosarin enantiomer could be detected for at least 20 min after i.v. administration of racemic cyclosarin to swine, whereas the (+)-isomer could not be detected in any sample even though the first blood draw was done about 2 min after poisoning (Reiter et al., 2007).

Monitoring the concentration−time profile of VX after i.v. challenge in swine, Reiter et al. (2011) also measured the concentration of its toxic hydrolysis product EA-2192 (*S*-[2(diisopropylamino)ethyl]methylphosphonothioic acid) by means of liquid chromatography-tandem MS (LC-MS/MS). This biotransformation was also observed in heparinized rat plasma in vitro, showing a notable stability, and should be considered for therapy of OP poisoning by VX.

52.3.12.2 Subcutaneous uptake

Subcutaneous exposure is often used as a substitute for respiratory exposure due to experimental difficulties in performing controlled inhalational poisoning (Benschop and de Jong, 2001). For example, we will note a typical study illustrating the s.c. behavior of soman (Fig. 52.3B). A C(±)P(±)-soman bolus injection in the scruff of a guinea pig neck resulted in a discontinuous C(−)P(−)-soman concentration−time profile following a monoexponential equation for the absorption phase and a biexponential fit for the distribution phase (Fig. 52.3B; Benschop and de Jong, 2001). The steep initial concentration increase in blood indicated rapid penetration through capillary walls. Maximum concentration was not reached until 7 min after soman injection, yielding an absorption half-life of about 3.5 min. Despite some certain diastereomer-specific differences [decreased bioavailability of C(+)P(−)-soman], both P(−)-forms exhibited comparable kinetic behavior. Bioavailability was found to be 70%−80% when comparing the corresponding area under the curve (AUC) to that of i.v. injection. Very similar to the i.v. characteristics mentioned previously, the toxic, more stable P(−)-isomers were detectable in blood for more than 3 h after exposure, whereas the less toxic P(+)-forms of soman could not be determined at any time. This is attributed to the high-hydrolyzing catalytic activity of PTEs in blood, skin, and other affected tissue (Table 52.3).

52.3.12.3 Percutaneous uptake

(±)-VX dissolved in isopropanol (1 LD_{50}) was applied p.c. to hairless guinea pigs (Fig. 52.3C; Van der Schans et al., 2003). Typically for p.c. exposure, concentration in blood increased very slowly, reaching its maximum between 3 and 4 h after challenge, followed by a slight decrease within the next 4 h. Despite that longer period of monitoring, the illustrated concentration−time profile only reflects the distribution and early elimination phases. This slow release is due to the formation of a depot under and in the skin, as well as to reduced hydrolytic degradation of VX in vivo. No prominent effects of stereoselective toxicokinetics were observed; therefore, the depicted curvature reflects the racemic mixture of analytes. Bioavailability of VX at 7 h was found to be very small compared to i.v. data (not exceeding 3%). The long-lasting elimination phase demonstrated high VX persistence in the organism, resulting in threatening concentrations with acute toxicological relevance for a longer period. Such information is important for therapeutic treatment, pointing out the necessity of long-time oxime infusion.

52.3.12.4 Respiratory uptake (nose-only model)

As pointed out in Section 52.6, respiratory uptake is the most likely route of exposure for G agents, causing 70%−80% absorption in the upper respiratory tract. The sophisticated exposure model of the nose-only design applied to guinea pigs challenged the laboratory animals free of potential distortions derived from simultaneous ocular or p.c. uptake, as occurs in vapor chambers (Langenberg et al., 1998a).

The toxicokinetics caused by administration of C(±)P(±)-soman to guinea pigs documented a discontinuous process of monoexponential function for the absorption phase followed by a biexponential fit for distribution and elimination (Fig. 52.3D). C(−)P(−)-soman was detected in blood as early as 30 s after challenge, and a short-term maximum was reached immediately after terminating the 8-min exposure period (Fig. 52.3D; Benschop and de Jong, 2001). Nevertheless, some depot formation might also occur when applying higher doses, causing maximum levels after completion of exposure. The concentration curvature of C(+)P(−)-soman showed a similar profile, but with consistently smaller concentrations, which is due to a higher degree of enzymatic hydrolysis (Table 52.3). In contrast, less toxic C(−)P(+)-soman was detected in very small concentrations during the exposure phase exclusively, whereas C(+)P(+)-soman was not detected at all. Curvatures for S_P-(−)-sarin appeared to be very similar in terms of time for first appearance in blood and for reaching maximum concentration.

These selected representative examples indicate that concentration−time profiles are variable despite common underlying basic chemical reactions of hydrolysis and adduct formation. Despite improving medical treatment of nerve agent poisoning, the concurrence of numerous physiological and pathophysiological parameters should be understood. Therefore, establishment of a descriptive and predictive model is important for medical defense of OPCs.

52.3.13 Mathematical simulation for prediction of nerve agent toxicokinetics

As is obvious from the huge number of parameters affecting toxicokinetic behavior (e.g., route of administration, nature of OPCs, hydrolyzing and bioscavenging enzymes,

proteins, and distinct compartments for distribution and species specificities), mathematical modeling of this complex situation is a big challenge. Based on numerous experimental data on soman toxicokinetics, Sweeney et al. (2006) introduced a model described by mathematical algorithms that allows the prediction of concentration−time profiles evoked by i.v., s.c., or inhalational soman uptake (≥1 LD_{50}) in common laboratory animals (i.e., rat, guinea pig, and marmoset). This physiologically based pharmacokinetic model (PB/PK) benefits from the combination of relevant pharmacokinetic basics and soman-specific experiences, realizing both an excellent degree of confidence for theoretical and laboratory data, as well as extrapolation to other species. This concept is a further development of an initial model from the same working group introduced by Langenberg et al. (1997). A more detailed description of these models would go beyond the scope of this chapter. Readers are referred to the sources in the reference list at the end of the chapter.

52.3.14 Bioanalytical techniques relevant to toxicokinetics

Elaboration of nerve agent toxicokinetics requires sophisticated analytical tools to detect and, if possible, quantify the free toxicants, as well as adducts with proteins and enzymes. Analysis of OP nerve agents has been performed by capillary electrophoresis (CE), biosensors, MALDI-MS, desorption electrospray ionization MS (DESI MS), ion mobility time-of-flight MS (IM-TOF MS), nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-ultraviolet (LC-UV), gas chromatography (GC), and many more techniques (Hooijschuur et al., 2002; John et al., 2008).

52.3.14.1 Determination of nerve agents

In contrast to those rather unusual methods, GC coupled to diverse detection systems [e.g., flame ionization detector (FID), nitrogen−phosphorus detector (NPD), flame photometric detector or mass spectrometer, as well as LC methods] represent the most common techniques for OP determination, especially for biological samples. These methods offer high resolution, sufficient limits of detection, good reproducibility, and robust hardware devices. For more detailed information, readers are referred to other review articles (e.g., Hooijschuur et al., 2002; John et al., 2008).

Analysis becomes much more complex when stereoisomers are quantified separately (Fig. 52.1). Enantiomers cause identical detector responses in NPD, FID, or MS. Therefore, chiral separation systems are required to overcome these detector limitations. Despite enormous progress in separation media and detector systems within the last two decades, the number of reports on chiral analysis of nerve agents valuable for toxicokinetic studies is still very small. Chiral separations make use of special chromatographic columns modified with chiral ligands.

Isomers of soman were separated by GC on a Chirasil-L-Val column, but lacked baseline separation (Benschop et al., 1981, 1985; Li et al., 2003a,b). In contrast, (+)-sarin and (−)-sarin could be completely separated by the same column (Benschop and de Jong, 2001). Another study presented a modified method using a Chiraldex gamma-cyclodextrin trifluoroacetyl GC-column coupled to an electron impact (EI)-MS, which enabled sufficient baseline separation of all four stereoisomers of soman (Smith and Schlager, 1996; Yeung et al., 2008).

Apart from the Chirasil-L-Val method, sarin enantiomers were also separated by a two-dimensional GC technique on chiral Cyclodex B material prior to NPD monitoring (Spruit et al., 2000, 2001). Additional GC-based approaches allowed baseline separation of cyclosarin enantiomers on a GAMMA DEX column monitored by EI-MS (Reiter et al., 2007), separation of VX stereoisomers on hydrodex-β-TBDAc (β-cyclodextrin; Reiter et al., 2008), and separation of tabun enantiomers on BetaDex 225 coupled to APCI-MS (Tenberken et al., 2010). VX enantiomers were also chromatographed on a Chiracel OD column by LC coupled to an electrochemical detector, yielding a lower limit of quantification of about 10 ng/mL blood (Van der Schans et al., 2003). Another LC method coupled with MS/MS detection for VX made use of CHIRALCEL OD-H and CHIRAL AGP columns (Reiter et al., 2008).

52.3.14.2 Detection of enzyme and protein adducts of nerve agents

In contrast to the measurement of free agents, the qualitative detection of protein adducts is a very novel approach that came about by overwhelming technical progress in bioanalytical mass spectrometry. Electrospray ionization (ESI) and MALDI as soft ionization methods for mass spectrometric detection are highly valuable for the detection of biomacromolecules like DNA, peptides, and proteins (Schulz-Knappe et al., 2001; John et al., 2004, 2005). Therefore, these technologies are also favorable for the analysis of proteins interacting with nerve agents. Consequently, a number of approaches have been published that identify and characterize these adducts. Typically, protein and enzyme adducts are first isolated from complex biological matrices (e.g., blood) using affinity chromatographic methods; subsequently, they are subjected to enzymatic cleavage by adding selected proteases. The resulting internal peptide cleavage products containing the derivatized phosphylated) residues are chromatographically separated and analyzed by modern

TABLE 52.5 Physicochemical properties of most common vesicant agents.

Agent	CAS no.	NATO code	MW (g/mol)	Melting point (°C)	Boiling point (°C)	Vapor pressure (mbar)	Water solubility (mg/L)	Hydrolysis rate, $\tau_{1/2}$ (h)	log *P* (—)
Sulfur mustard	505-60-2	HD	159.1	14	217	0.147 (25°C)	0.684[a]	14.7 (20°C)	1.37
Lewisite	541-25-3	L	207.3	−18	190 (decomposing)	0.773 (25 °C)	500	Rapid, n.a.	n.a.

log *P*, octanol:water partition coefficient; MW, molecular weight; n.a., not available; $\tau_{1/2}$, period of half-life for hydrolysis.
[a]*Seidell (1941)*.
Source: Data are taken from Munro, N.B., Talmage, S.S., Griffin, G.D., 1999. The sources, fate, and toxicity of chemical warfare agent degradation products. Environ. Health Perspect. 107, 933–974 unless otherwise noted.

mass spectrometry. Using sophisticated MALDI-MS techniques, this general procedure allowed the identification of albumin adducts (Li et al., 2007, 2008; John et al., 2010), as well as the adducts of BChE and their aged products (Jiang et al., 2013). LC-ESI MS was applied to analyze adducts of albumin (Peeples et al., 2005; Williams et al., 2007), BChE (Noort et al., 2006; Tsuge and Seto, 2006; John et al., 2015), CarbE (Peeples et al., 2005), keratins, tubulin, actin, and transferrin (Grigoryan et al., 2009; Schopfer and Lockridge, 2019). For a more detailed description, see John et al. (2008), Schopfer and Lockridge (2012), and Black and Read (2013).

For diagnostic detection of BChE adducts of common G- and V-type nerve agents, enhanced sample throughput was achieved by automated processes in 96-well plate format, extracting plasma by immunomagnetic separation (Knaack et al., 2012). An alternative method, based on the principle of a sandwich enzyme-linked immunosorbent assay (ELISA), was presented by Wang et al. (2011) to determine OP adducts in complete BChE. Modern mass spectrometry-based methods were successfully applied to prove a case of fatal sarin poisoning in the violent conflict in the Syrian Arab Republic in 2015 (John et al., 2018a,b).

This summary underlines the great importance of modern analytical techniques to unraveling pathophysiological situations at the molecular level and to supporting toxicokinetic studies by discovering the most relevant protein-binding elimination processes.

52.4 Vesicants

52.4.1 Sulfur mustard

52.4.1.1 Overview of sulfur mustard

Sulfur mustard is a blistering or vesicating agent that primarily incurs damage on organs that come into immediate contact with either its liquid or vaporous form. Even low-dose dermal and respiratory exposure to the agent results in systemic absorption of sulfur mustard, which subsequently may cause additional systemic damage (Kehe and Szinicz, 2005; Steinritz et al., 2016).

Unfortunately, sulfur mustard has been used in acts of chemical warfare throughout the 20th century, from World War I to the attacks of Saddam Hussein's former Iraqi regime against Iran and even Kurdish civilians or the repetitive use of sulfur mustard in the Syria crisis starting in 2013. The simplicity of the agent and its synthesis combined with its devastating medical, psychological, and socioeconomic effects, along with the fact that no causative therapy has yet been established, may convince future aggressors (both state and nonstate parties) to use this agent in their attacks. This, in turn, necessitates medical research efforts, including toxicodynamic and toxicokinetic studies, to develop countermeasures against the effects of sulfur mustard. Table 52.5 displays the basic physicochemical properties for sulfur mustard, which constitute the fundamental reason behind many of its toxicokinetic properties. Data for lewisite, another vesicant agent described Section 52.44.2, are also shown in this table.

52.4.1.2 Toxicity of sulfur mustard

Fig. 52.4 depicts the basic chemical mechanism by which sulfur mustard incurs its primary damage to biological molecules, which results in subsequent damage to cells, tissues, and organs. Sulfur mustard forms an intermediate sulfonium ion that further transforms into a carbenium ion, a strong electrophile capable of reacting with nucleophile targets, primarily in DNA and RNA. Examples include the N7 in guanine (61%; Ludlum et al., 1994), the N3 in adenine (6.6%), and the O6 in guanine (0.1% for chloroethylthioethene) (Ludlum et al., 1986). Even though the latter is considerably less frequent, it is of significant concern, as the repair enzyme (O6-alkylguanine-DNA-alkyltransferase) is not capable of reversing this reaction. Therefore, DNA mutation due to mispairing may occur

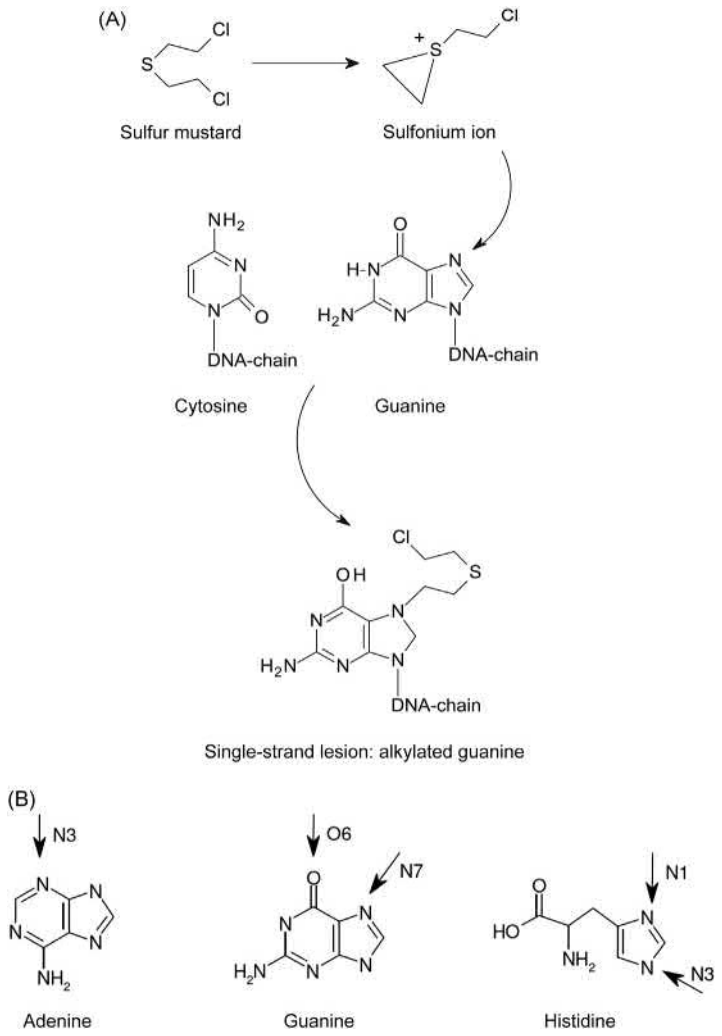

FIGURE 52.4 Sulfur mustard, its structure, mechanism of action, and targets of adduct formation. (A) Mechanism of reaction of sulfur mustard and nucleotide guanine. (B) Sites of alkylation by sulfur mustard. Arrows mark identified targets in nucleotides and amino acid histidine.

during DNA replication, which is proposed to be the origin of subsequent cancer formation (Ludlum et al., 1986). Because of its bifunctional character, sulfur mustard also forms interstrand DNA cross-links, in particular from one guanine to another (17%). It is estimated that a 100-μM concentration of sulfur mustard accounts for 0.28 cross-links per 10,000 DNA bases (Shahin et al., 2001). Remarkably, adipose tissue seems more sensitive to sulfur mustard in means of DNA adduct formation. Here, cross-links were more abundant compared with in nonadipose tissue (Wang et al., 2015). Moreover, more recent studies, that investigated sulfur mustard DNA adduct formation in both cell cultures and animal experiments, revealed a dose-dependency regarding the formation of N7-HETEG, N3-HETEA, O6-HETEG, and Bis-G (Yue et al., 2014). Furthermore, cross-links between N7 in guanine and glutathione (GSH) were described by Batal et al. in murine organs (skin, brain, and lung) after percutaneous in vivo exposure of mice to sulfur mustard (Batal et al., 2015). GSH adducts were found in quantities similar to the Bis-G product (Batal et al., 2015). Membrane-bound proteins and enzymes may undergo alkylation in the presence of sulfur mustard (Kehe and Szinicz, 2005). Recently it was shown that chemosensing TRPA1 channels are targeted by sulfur mustard and that TRPA1 activation contributes to cytotoxicity (Stenger et al., 2015). The exact activation mechanism is still not fully understood. Covalent modification of intracellular cysteine residues, but also interaction between sulfur mustard and extracellular or transmembrane parts, may occur (Stenger et al., 2017) Fig. 52.4 depicts targets of alkylation in both DNA bases and amino acids. As sulfur mustard-induced DNA damage has the most significant impact on the cell's short- and long-term survivability, it is DNA alkylation that produces the most significant toxic effects, whereas effects from protein alkylation are usually visible only in the presence of high concentrations of sulfur mustard (Peters, 1947; Lodhi et al., 2001). As systemic

concentrations will always be several orders of magnitude below local concentrations at the site of immediate contact, the systemic toxic effects of sulfur mustard are also most likely to result from DNA alkylation. DNA alkylation produces the most evident effects in proliferating cells; tissues with rapidly proliferating cells, such as bone marrow, suffer the most obvious damage. Toxic effects are similar to side effects from alkylating antineoplastic drugs (Dacre and Goldman, 1996). [Actually, the first antineoplastic drug was developed from a structural analog of nitrogen mustard—see Goodman et al. (1984).] Those effects include nausea, vomiting, fever, fatigue, apathy, and loss of appetite. Bone marrow toxicity results in an initial leukocytosis, which is soon followed by leukopenia, thrombopenia, eosinopenia, and subsequent anemia (Dacre and Goldman, 1996).

Specific toxic effects of sulfur mustard have also been reported in the CNS and may range from agitation to seizures (Balali-Mood and Navaeian, 1986). In addition to these acute CNS symptoms, long-term neurological and neuropsychological complications of sulfur mustard and lewisite were also reported (Isono et al., 2018).

Sulfur mustard-induced DNA alkylation is regarded as exclusively causal for toxicity. However, it was demonstrated that sulfur mustard is able to provoke immediate behavior responses along with fast changes in the electrical field potential (EFP) of neurons in the insect species *Blaptica dubia*, suggesting that lesions of DNA are probably not the only effect of alkylating compounds (Popp et al., 2018).

52.4.1.3 Invasion

Significant amounts of sulfur mustard may be absorbed from skin that has been in direct contact with sulfur mustard. Vapors of sulfur mustard may also be absorbed through the respiratory system.

In theory, there are two additional pathways for systemic uptake of sulfur mustard: the surface of the eyes and the gastrointestinal system. They have, however, never been investigated for the following reasons: gastrointestinal exposure to sulfur mustard is a rare phenomenon and may only occur in cases of sabotage (deliberate food poisoning) or a considerable lack of basic chemical defense measures (i.e., food consumption in a contaminated area). The local effects of exposing the gastrointestinal system to sulfur mustard are severe and life-threatening; they include strong abdominal pain, bloody diarrhea and vomiting, and rupture of stomach or duodenum. Subsequent peritonitis is often fatal (Dacre and Goldman, 1996). In comparison to life-threatening local effects of gastrointestinal exposure, any related systemic effect would be of secondary importance.

As opposed to the rarity of gastrointestinal exposure, ocular exposure is frequent among the victims of sulfur mustard attack, and local symptoms of varying severity are likely to occur whenever the individual was not protected by a respirator (gas mask) at the time of attack (Solberg et al., 1997). As the eye is a very sensitive organ, ocular symptoms are often among the first signs of sulfur mustard exposure. Due to the close proximity of a large number of capillary vessels and the fact that the eye constitutes a relatively weak barrier, xenobiotic substances are often rapidly absorbed (Lama, 2005). As the surface of the eye is small in comparison to the skin and the respiratory tract, the total amount of sulfur mustard that may be absorbed from the eye's surface is low. Self-protective effects of the eye, such as blepharospasm and pronounced lacrimation, may further reduce the amount of an agent that may be absorbed through this pathway. The local effects of exposure appear very severe and are a primary reason for concern in victims of sulfur mustard exposure. Whereas blepharospasm and lid edema cause transitory loss of vision—already resulting in immense distress for victims—heavy exposure may result in permanently opaque cornea and blindness. In general, early effects in the eyes are treatable with therapy and have a relatively good outcome after several weeks. In comparison, any systemic effects from transocular absorption alone would be of only minor concern. It has to be emphasized that isolated ocular exposure on the battlefield or due to terrorist attacks utilizing sulfur mustard is exceedingly unlikely. Ocular exposure would inevitably be accompanied by cutaneous and respiratory exposure. The specific rate of absorption (i.e., the amount absorbed through a given surface over a specified time) may be lower, particularly in the case of initially intact skin. However, the larger surface of the skin (and possibly the respiratory tract) would result in an amount that constitutes the major fraction of sulfur mustard absorbed, which would subsequently determine the occurrence and severity of systemic effects. Research regarding the absorption of sulfur mustard has focused on two major pathways, which are described in further detail in the subsequent sections.

52.4.1.3.1 Percutaneous absorption

Penetration rates of liquid sulfur mustard were determined in vitro (71−294 $\mu g/cm^2/h$) on human skin with a Franz-type glass diffusion cell and correspond very well to in vivo data derived from human skin (60−240 $\mu g/cm^2/h$; Chilcott et al., 2000). Significant effort has been dedicated to confirm the validity of animal models by comparing data derived from these models with findings from in vitro human skin experiments. Chilcott et al. (2001) investigated absorption in in vitro models of pig-ear and heat-separated human skin. Absorption was determined at 411 ± 175 $\mu g/cm^2/h$ for pig-ear skin and 157 ± 66 $\mu g/cm^2/h$ for human skin, respectively. These data were considered to agree with earlier in vivo findings, even

though the authors cautioned that data on effectiveness of decontaminants were completely disparate between human and pig-ear skin. Logan et al. (1999) determined that sulfur mustard exposure through skin of the hairless guinea pig was 120 $\mu g/cm^2/h$.

Occlusion of the skin (i.e., covering the skin with a material that is impenetrable to air and moisture) can result in a dramatic increase in p.c. absorption. For example, in a study by Chilcott et al. (2002) using in vitro models of human skin, absorption rates in unoccluded controls ($4.41 \pm 1.90\ \mu g/cm^2/h$) increased to $538 \pm 193\ \mu g/cm^2/h$ under occluded conditions.

In contrast, Karvaly et al. (2008) showed that a commercially available barrier cream and a perfluoropolyether oil applied prior to exposure reduced p.c. uptake of sulfur mustard. In particular, perfluoropolyether oil was highly effective, preventing sulfur mustard exposure for a period of 20 min. A petroleum jelly ointment, however, had no protective effect, even when applied prior to exposure.

Benson et al. (2011) percutaneously exposed guinea pigs to ^{14}C-labeled sulfur mustard vapors ($525\ mg/m^3$) for 12 min, applied to three skin areas totaling $19.8\ cm^2$. A total of $29.8 \pm 5.31\ \mu g$ sulfur mustard per animal was absorbed, 90% of which remained in the skin. This result agrees with an in vitro study by Hattersley et al. (2008) using human skin samples, which confirmed that a depot of sulfur mustard existed for at least 24 h following dermal exposure, and estimated concentrations in skin are at least 20 times above the acutely toxic concentration.

Regarding airtight vapor cups frequently used for percutaneous sulfur mustard exposure studies, Dalton et al. (2006b) validated this approach and showed that equilibrium concentrations developed within 2 min after sealing the cups and were not significantly different from calculated saturated vapor concentration ($1{,}363\ mg/m^3$) when cups were placed on inert control surfaces. However, when cups were placed on samples of pig skin, percutaneous uptake of sulfur mustard lowered actual vapor concentrations to values significantly below saturation (i.e., to $592 \pm 246\ mg/m^3$ on a $10.15\ cm^2$ skin surface and $740 \pm 224\ mg/m^3$ on a $2.54\ cm^2$ skin surface). The authors validated the assumption that saturated vapor concentration could be used to calculate concentration−time products for percutaneous absorption studies, but they cautioned that, depending on the size of the reservoir and the skin surface, decreasing vapor concentrations had to be taken into account.

52.4.1.3.2 Respiratory absorption

Langenberg et al. (1998b) conducted inhalation exposure experiments in hairless guinea pigs. LCt_{50} was determined at $800\ mg\ min/m^3$. Following application of 1 LCt_{50} over 5 min, no unchanged sulfur mustard in blood was found (with a limit of detection of 5 pg/mL). Low concentrations (0.7 adducts per 10^7 nucleotides) of N7-guanine adducts of sulfur mustard were found in the lung. The concentration of N7-guanine adducts was also determined from various tissues along the respiratory tract. In fact, concentrations found were much larger, peaking at approximately 90 adducts per 10^7 nucleotides in the larynx and trachea. The concentration in the carina was lower (approximately 50 per 10^7 nucleotides), but it was still substantially higher than the abovementioned value in the lungs. The authors concluded that most of the sulfur mustard inhaled reacts in the upper airways rather than being absorbed. In animals with complex nasal systems (such as guinea pigs), only very minor fractions would reach the lung. However, they also pointed out that, in humans (along with other species with a less complex nasal system), the proportion of sulfur mustard reaching the lung may be larger.

Following inhalation of $3 \times LCt_{50}$ ($300\ mg/m^3$ over a period of 8 min), sulfur mustard was found in blood. Its peak concentration during the inhalation period was found to be approximately 5 ng/mL. This concentration soon declined. A mean value of 2 ng/mL was found even after 4 h. A mathematical model explaining the data during the distribution and elimination phase could not be developed. These findings were further complicated by the fact that in 2 of 12 animals, no sulfur mustard was found in any of the samples. No sulfur mustard was detected in any of the animals at 15 and 20 min (7 and 12 min postexposure, respectively), before reappearing in most of the animals at the abovementioned concentration of 2 ng/mL. The latter finding may possibly be explained by the hypothesis that the early peak in sulfur mustard concentration is due to direct inhalation (afterward, concentration declines to levels below the limit of detection), whereas the second, long-term increase in sulfur mustard concentration may be attributed to sulfur mustard absorbed from depots in the upper airways. Fig. 52.5 depicts the concentration over time, following the respiratory exposure to $3 \times LCt_{50}$ of sulfur mustard. For comparison, concentration over time after i.v. application is also depicted. The authors concluded that toxicity from sulfur mustard inhalation was due to its local, rather than its systemic, effects.

Benson et al. (2011) presented a respiratory exposure study using ^{14}C-labeled sulfur mustard. Anesthetized rats with transorally placed tracheal catheters were exposed to $250\ mg\ ^{14}C$ sulfur mustard vapor/m^3 for 10 min. A total of $18.1 \pm 3\ \mu g$ sulfur mustard per animal was absorbed. Within 2 h postexposure, inhaled sulfur mustard was distributed and more than 70% were deposited in the carcass and pelt.

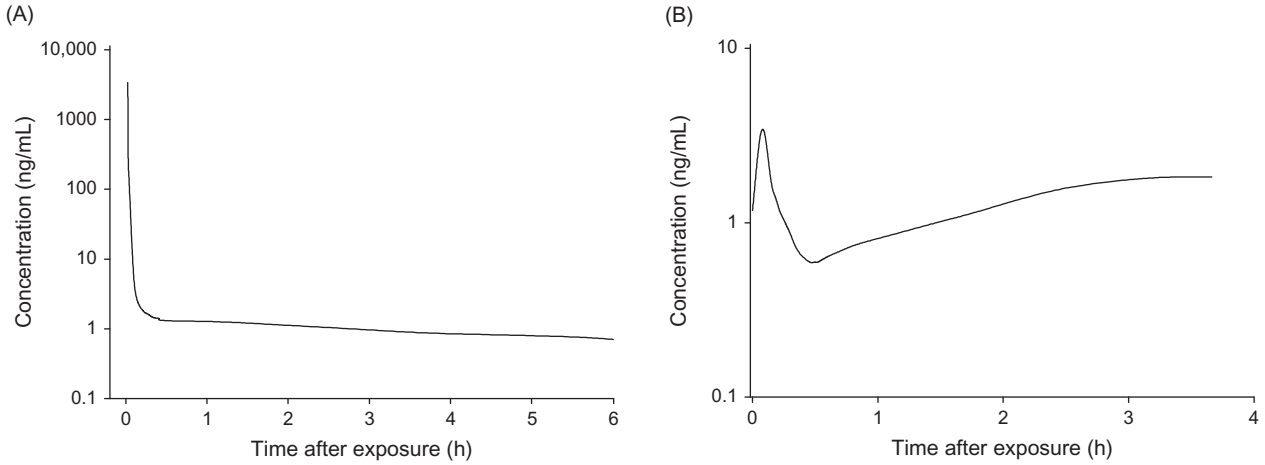

FIGURE 52.5 Concentration over time, following i.v. and respiratory exposure to sulfur mustard in the guinea pig. (A) Decline of sulfur mustard exposure after i.v. injection. (B) Concentration over time after respiratory exposure: initial increase in the inhalation phase, followed by a decline and a secondary increase; concentration of approximately 2 ng/mL is sustained for 4 h.

52.4.1.4 Distribution

Sulfur mustard is a strongly lipophilic substance that may accumulate in fatty tissues and has been detected at autopsy in a patient who died 7 days postexposure. Detailed data, as published by Drasch et al. (1987), are displayed in Table 52.6. This case report observation was affirmed by animal experiments using rats (Xu et al., 2017).

Obviously, these findings confirm the theoretical assumption that the lipophilic properties of sulfur mustard result in a distribution, primarily in lipophilic tissues. High concentrations found in the brain may also explain why the CNS is one of the organs exhibiting systemic effects of sulfur mustard poisoning, even though it is not a site of rapidly proliferating cells.

It should be noted that some authors have questioned the findings by Drasch et al., considering the absolute concentrations excessively high and therefore unlikely. However, as high-dose sulfur mustard poisoning is a rare event, it is nearly impossible to verify or falsify the data. One might argue that the amounts of sulfur mustard described may not even permit short-term survival. However, if most of an agent entered the organism via p. c. absorption, there is a possibility that large amounts of the agent actually had been absorbed and formed a depot without resulting in instant death, but ultimately leading to death 7 days later. Whether or not the absolute values are accurate, they at least give an impression of the distribution of sulfur mustard within an organism that correlates with the agent's lipophilic properties.

More recent in vitro experiments using human skin have confirmed the presence of unhydrolyzed sulfur mustard in the lipophilic stratum corneum and the upper epidermis. At 24 h post-exposure, the distribution ratio between epidermis and dermis has been determined to be 62%–38%. Chilcott et al. (2000) also suggested that efforts to remove or neutralize the agent from these deposits might have a clinical benefit for the patient.

These findings suggest that, despite the presumably brief half-life calculated (see Section 52.44.1.7), sulfur

TABLE 52.6 Content of sulfur mustard in the tissues of a deceased victim.

Tissue/organ	Concentration (mg/kg)
Fat	15.1
Skin with subcutaneous fatty tissue	11.8
Brain	10.7
Skin	8.4
Kidney	5.6
Liver	2.4
Cerebrospinal fluid	1.9
Muscle	3.9
Spleen	1.5
Blood	1.1
Lung	0.8
Urine	Not detected
Blister fluid	Not detected

Source: Data are according to Drasch, G., Kretschmer, G., Kauert, L., 1987. Concentration of mustard gas [bis(2-chloroethyl)sulfide] in the tissues of a victim of a vesicant exposure. J. Forensic Sci. 32, 1788–1793.

mustard may be present in an organism for a significantly longer period of time, necessitating measures for medical personnel providing therapy and an awareness for the possibility of secondary blister formation, even 30 days post-exposure (Balali-Mood and Hefazi, 2005), as well as efforts to remove these deposits. In particular, the use of laser debridement (Graham et al., 2008) and mechanical dermabrasion (Rice, 2008) has been recommended to facilitate the healing process after dermal exposure to sulfur mustard. The effects may be partly attributed to the removal of epidermal depots of sulfur mustard.

As mentioned previously, Benson et al. (2011) confirmed that 90% of percutaneously absorbed sulfur mustard was deposited in skin, whereas more than 70% of absorbed sulfur mustard was distributed to the carcass and pelt after respiratory exposure. The distribution of sulfur mustard is highly dependent on the original route of exposure. It was confirmed that a considerable amount of sulfur mustard was still present in deep lipophilic compartments even after the end of exposure.

52.4.1.5 Biotransformation

While the previously discussed findings demonstrate the stability of sulfur mustard in lipophilic tissues, the agent is rapidly hydrolyzed whenever situated in an aqueous compartment (Vycudilik, 1987). Thiodiglycol (TDG) is the primary hydrolysis product, in which the chlorine atoms have been replaced by hydroxyl groups. Karvaly et al. (2008) used subcutaneous microdialysis to monitor TDG in rats exposed to sulfur mustard. Peak concentrations of 7.2−21.7 nmol/L TDG were found, following percutaneous exposure to 2 μM sulfur mustard.

TDG may undergo oxidation to TDG sulfoxide, which is conjugated with glutathione to form 1,1′-sulfonylbis [2-*S*-(*N*-acetylcysteinyl)ethane]. Following the β-lyase pathway, the 1,1-sulphonylbis[2-(methylsulfinyl)ethane] and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane can be formed (Black and Read, 1995). Fig. 52.6 summarizes the pathways of sulfur mustard biotransformation. No sulfo- or glucuronyl-conjugates were detected in urine after i.v. administration of sulfur mustard (Maisonneuve et al., 1993).

Halme et al. (2011) presented a method for the efficient stable isotope dilution LC-HESI-MS/MS method for verification of β-lyase biotransformation products in human urine after sulfur mustard exposure. The β-lyase products 1,1′-sulfonylbis-[2-(methylsulfinyl)ethane] (SBMSE) and 1-methylsulfinyl-2-[2-(methylthio) ethylsulfonyl]ethane (MSMTESE) were successfully detected at a concentration of 4 ng/mL, whereas the limit of quantification was established at 10 and 11 ng/mL for SBMSE and MSMTESE, respectively.

Within cells, sulfur mustard forms adducts with DNA, primarily those described above. Adducts can also be formed with nucleophilic sites of amino acids and proteins. Byrne et al. (1996) demonstrated that sulfur mustard, with two highly reactive groups, was able to form protein cross-links between cysteine residues and assumed that protein cross-link formation may actually contribute to sulfur mustard toxicity. Contrary to DNA adducts, there is no specific mechanism to reverse protein adduct formation. For this reason, there is a strong forensic interest in the detection of protein adducts of sulfur mustard, as these may provide evidence of sulfur mustard exposure for prolonged periods after an incident.

Capacio et al. (2008) developed a method to detect sulfur mustard adducts in plasma proteins by hydrolyzation (which produced TDG) and subsequent derivatization of TDG, followed by GS/MS analysis. The method was successfully applied to determine sulfur mustard adducts in rat plasma, following respiratory exposure to sulfur mustard.

A number of adducts to amino acid residues have been identified (Noort et al., 1996; Black et al., 1997a,b; John et al., 2019; Siegert et al., 2019). Several histidine, glutamic acid, aspartic acid, cysteine, methionine, tryptophan, and valine residues were found to be alkylated. While N1 and N3 histidine adducts were found to be most abundant, it was the alkylated *N*-terminal valine adducts of hemoglobin that were most useful for subsequent quantification. See Section 52.4.6.2 for analytical details.

Noort et al. (2008) investigated the persistence of sulfur mustard adducts to albumin and hemoglobin in rats. The albumin adduct (*S*-2-hydroxyethylthioethyl)-Cys-Pro-Tyr was detectable up to 7 days after exposure, while the adduct to the *N*-terminal valine in hemoglobin was still detected after 28 days. The decrease in the adduct concentration corresponded with the albumin half-life and the lifetime of the rat erythrocyte, respectively. The respective adduct of human serum albumin was detected for at least 29 days after exposure in three cases of accidental human poisoning with sulfur mustard vapor (Steinritz et al., 2016).

Following two simultaneous cases of accidental human exposure to sulfur mustard, Smith et al. (2008) investigated the concentration of cysteine-34 adduct to albumin and adducts to glutamic and aspartic acids of plasma proteins. In the case of a more severely exposed patient who required hospitalization, both adducts were detected over a 42-day period, although they decreased by approximately 75% toward the end of that time. In a second patient who had developed a single, small blister, the albumin adduct was found during a 6-day period post-exposure. John et al. (2019) reported on four cases of human poisoning as a result of a mortar attack in the

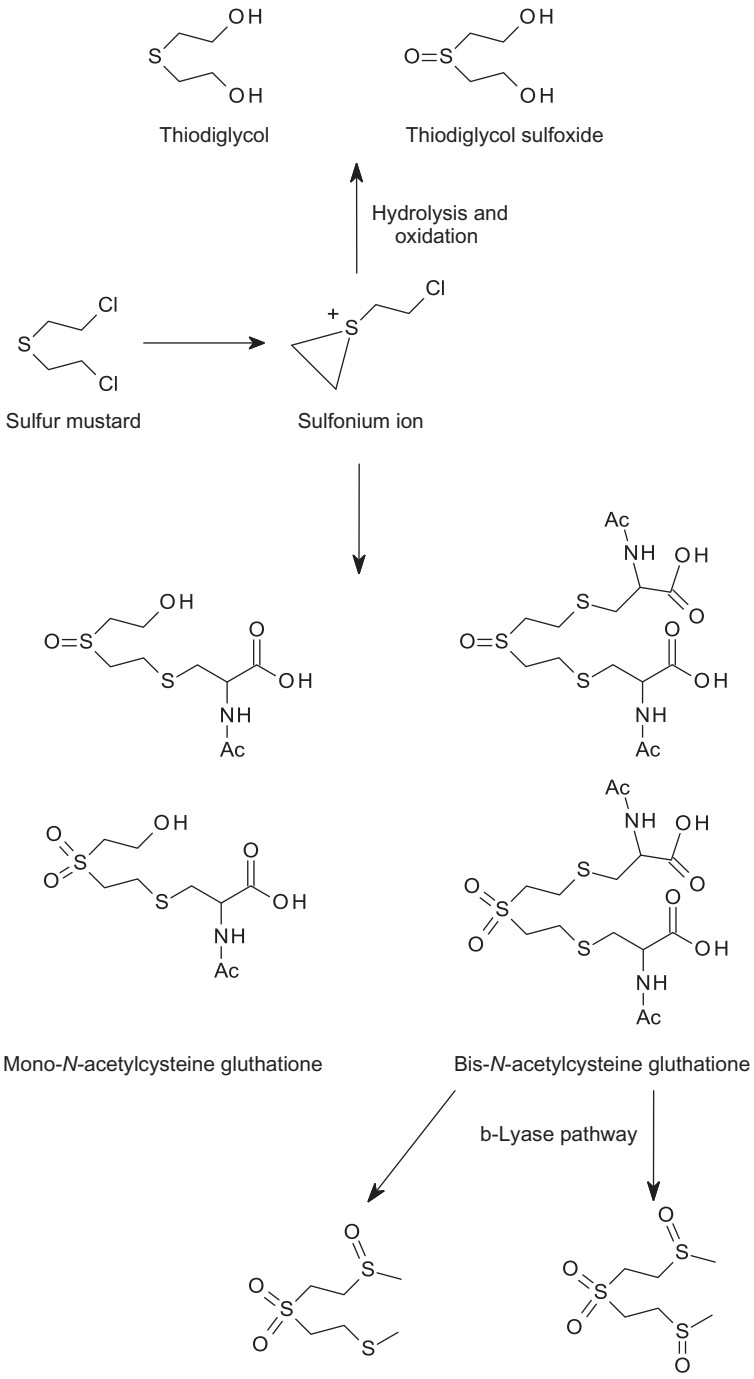

FIGURE 52.6 Biotransformation of sulfur mustard.

Middle East. Exposure to sulfur mustard was proven by detection of diverse albumin adducts even though blood drawing from poisoned persons was done 15 days after exposure (John et al., 2019).

Recently, local adduct formation after cutaneous exposure to sulfur mustard has been a focus of investigation in an effort not only to establish new forensic methods, but also to better understand vesication, the most evident symptom of sulfur mustard exposure via the skin. Adducts of sulfur mustard to cytokeratin types I and II, actin stratifin, and galectin-7 were successfully identified by Mol et al. (2008) in sulfur mustard-exposed human epidermal keratinocytes. Sulfur mustard adducts to actin, annexin A2, and keratin 9 were also found in HaCaT cells (immortalized keratinocytes) by Sayer et al. (2009).

52.4.1.6 Elimination

Following i.v. application of ^{14}C-labeled sulfur mustard in rats, 80% of the radioactivity administered was

excreted via the renal pathway. Fecal excretion amounted to less than 3% (Maisonneuve et al., 1993). A study by Benson et al. (2011) in rats and guinea pigs, following respiratory and percutaneous exposure to ^{14}C-labeled sulfur mustard, confirmed predominantly renal excretion. Biotransformation products excreted in urine after accidental human exposure included TDG, TDG sulfoxide, and the bis-mercapturate of mustard sulfone (Barr et al., 2008). When comparing the concentrations of TDG and its sulfoxide, the latter was found in concentrations twice as high. When p.c. exposing weanling pigs to sulfur mustard, Graham et al. (2000) found peak levels of TDG either in the samples drawn after 6–8 h or in other cases after 24–48 h. Findings were considered to be in agreement with earlier data from rodent species and cases of accidental human exposure.

It is worth noting that background levels of TDG and TDG sulfoxide have been found in the urine of healthy individuals never exposed to sulfur mustard. For this reason, their validity as unambiguous markers for sulfur mustard exposure has been questioned. Black and Read (1995) suggested the determination of β-lyase pathway biotransformation products, which in the urine of exposed patients were found at concentrations similar to those of TDG sulfoxide, but were not found in unexposed individuals.

52.4.2 Lewisite

52.4.2.1 Overview of lewisite

Lewisite (2-chlorvinyldichlorarsin) is another vesicant. It was first synthesized and described by the Belgian priest and chemist Julius Arthur Nieuwland (Nieuwland, 1904). Unlike sulfur mustard, there has never been a documented use of this substance in armed conflict. During World War I, the American military chemist Winford Lee Lewis suggested and initiated its development into a chemical weapon, but due to the 1918 armistice in Europe, it was not used on the battlefield (Vilensky and Redman, 2003).

Lewisite remains a concern because its physical properties (in particular a melting point of −18°C; see Table 52.5) might facilitate its use in cold climates. A belligerent party willing to use chemical weapons might decide that lewisite would provide a "unique" capability to wage chemical warfare even in winter or in mountainous regions. Large stockpiles of the agent were abandoned by the Imperial Japanese Army during its retreat from China in the latter stages of World War II, creating a chemical hazard that persists decades after the war (Hanaoka et al., 2006). Table 52.5 summarizes the physicochemical properties of lewisite.

52.4.2.2 Toxicity of lewisite

The dominant element in lewisite structure is arsenic, which is able to react with sulfhydryl groups of various enzymes, disabling the enzyme in the process (Goldman and Dacre, 1989). Fig. 52.7 depicts the structure of lewisite and its toxic mechanism.

Lipoic acid is particularly susceptible to this reaction and one of the most evident consequences is the inhibition of the enzyme pyruvate dehydrogenase (PDH), rapidly disabling the cell's metabolism of glucose and fatty acids. The resulting energy deficiency may lead to swift, necrotic cell death. In comparison to sulfur mustard, the latency period (from exposure to first signs and symptoms) is significantly shorter, and lewisite injuries have been described as extremely painful from an early stage. This may be explained by activation of TRPA1 channels by lewisite. Moreover, it is a much more lethal agent and has a large systemic toxicity; 0.5 mL of the agent may produce systemic effects, whereas 2 mL (approximately 3.6 g) can be fatal (Marrs et al., 1996).

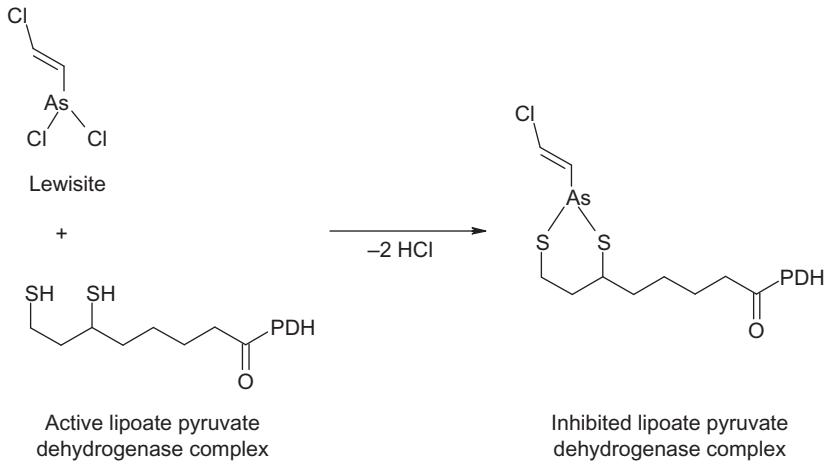

FIGURE 52.7 The structure and mechanism of action of lewisite. Lewisite forms covalent bonds with lipoic acid, inactivating the enzyme PDH.

52.4.2.3 Invasion

52.4.2.3.1 Percutaneous absorption

Precise data [i.e., a diffusion coefficient expressing the p.c. absorption of lewisite (amount absorbed per area and time)] is not available. However, Inns and Rice (1993) conducted p.c. toxicity studies in rabbits and determined the LD_{50} to be 5.3 mg/kg (3.5−8.5 mg/kg, 95% confidence interval). The exposed area was 2 cm^2, and exposure lasted for 6 h.

Inns et al. (1990) had also determined the LD_{50} of i.v. lewisite administration to be 1.8 mg/kg (1.6−2.1 mg/kg 95% confidence interval). It can be concluded that by exposing 2 cm^2 of rabbit skin to a dose of 5.3 mg/kg for 6 h, a dose producing the equivalent effect of 1.8 mg/kg is absorbed. No further calculations that might exaggerate the reliability of available data shall be conducted here.

King et al. (1992) conducted lewisite absorption studies on isolated perfused porcine skin flaps, using lewisite concentrations from 0.07 to 5.0 mg/mL. Blister formation was observed, and at higher lewisite concentrations, there was a pronounced increase of lactate dehydrogenase in blister fluid, a sign of necrotic cell death. Cellular glucose utilization was decreased at the same time, underscoring the detrimental effect of lewisite on cellular energy metabolism. At the highest concentration of 5 mg/mL of lewisite, arsenic was detected in venous blood from the perfused skin preparation. At this concentration, systemic uptake of lewisite or its degradation products and toxic effects resulting thereof are to be expected.

52.4.2.3.2 Respiratory absorption

The LCt_{50} of lewisite in humans has been estimated at 1500 mg min/m^3 (ATSDR, 2007), although no experimental data have been cited. Considering its pronounced local effects, lethality can at least partly be attributed to local effects of lewisite on the respiratory tract.

52.4.2.4 Distribution

High distribution volumes per kilogram indicate extensive distribution in tissues, due to the lipophilicity of the substance. In a rabbit model, more than sevenfold more of the substance was found in some tissues (e.g., the liver, lung, and kidneys) compared to blood concentrations. That ratio was maintained over the sampling period, that is, for at least 96 h (Snider et al., 1990).

52.4.2.5 Biotransformation

Once incorporated, unbound lewisite is quickly hydrolyzed. Its predominant biotransformation product is 2-chlorovinylarsonous acid (CVAA; Fig. 52.8). Analytical methods to confirm lewisite exposure have focused on the detection and quantification of CVAA, at least in the past. However, Noort et al. (2002) also pointed out that, due to the high affinity of arsenic toward sulfhydryl groups, adducts of lewisite/CVAA and cysteine residues of proteins are formed. In an in vitro study, incubating 14C-labeled lewisite with human blood samples, 90% of lewisite was found in erythrocytes, whereas 25%−50% of

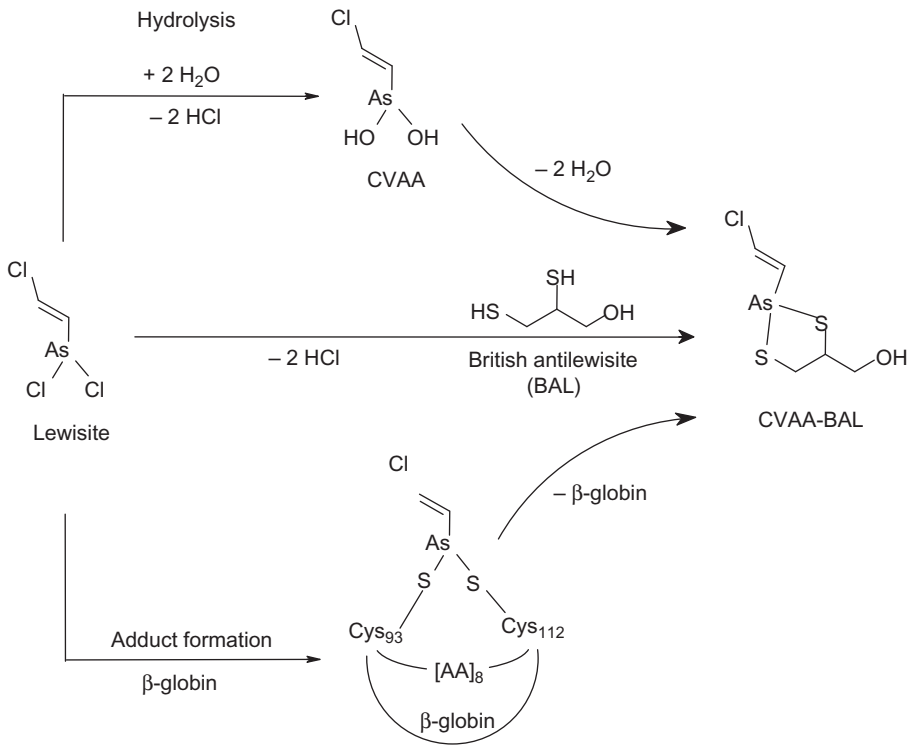

FIGURE 52.8 Lewisite biotransformation, adduct formation, and its reversal.

arsenic was bound to globin. From these protein adducts, CVAA can be released to form an adduct with the antidote British antilewisite (BAL; Fidder et al., 2000). The authors were also able to identify a specific protein adduct of lewisite formed with cysteine residues 93 and 112 of β-globin. See Section 52.46.2 for analytical details. Fig. 52.8 summarizes the biotransformation and reversal of adduct formation by BAL.

52.4.2.6 Elimination

Snider et al. (1990) determined the elimination of lewisite from rabbits after p.c. injection. The half-life was determined, ranging from 55–75 h. A clearance of 120 mL/h/kg was found. These findings only describe the overall elimination of arsenic from the organism following a lewisite exposure.

In vivo, unbound CVAA is quickly excreted via the renal pathway and cannot be detected in urine samples taken later than 12 h postexposure. The biological half-life of protein adducts is much longer: in blood samples taken 10 days postexposure and treated with BAL, Fidder et al. (2000) were still able to release 10% of the CVAA-BAL concentration found on day 1. Protein adducts of CVAA play an important role in the verification of potential lewisite exposure.

52.4.2.7 Bioanalytical techniques for quantification of vesicants

52.4.2.7.1 Determination of vesicants and direct biotransformation products

When Drasch et al. (1987) determined the concentration of sulfur mustard in tissues of a deceased victim, they had to employ a combination of dichloromethane extraction and a thin-layer chromatography cleanup on silica plates, followed by derivatization with gold chloride and quantification by electrothermal atom absorption spectroscopy.

Vycudilik (1985) had already used gas chromatography-mass spectrometry (GC-MS) to confirm the presence of sulfur mustard in urine samples. Sodium chloride was added to the sample to facilitate extraction and slow down hydrolysis in the aqueous sample, and then the analyte was extracted with diethylether. The solvent was evaporated and the residue dissolved in methylene chloride. After purification—by shaking the sample solution for 1 h with silicagel—the solvent was again evaporated. The residue was again dissolved in methylene chloride and used for chromatography.

To detect TDG, TDG sulfoxide, and their acid-labile esters, Black and Read (1991) used $TiCl_3$ reduction, converting these products into single analyte TDG. TDG was then converted to its bis(pentafluorobenzoyl) derivative and quantified by GC-MS using negative ion chemical ionization. TDG sulfoxide could also be extracted directly using solid-phase extraction, followed by a Florisil cleanup. Derivatization and quantification were conducted as described previously. $TiCl_3$ was also used by Daly and O'Hehir (2007) to reduce the β-lyase pathway biotransformation product 1,1-sulfonylbis[2-(methylsulfinyl)ethane] to 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE). This was followed by automated solid-phase extraction and LC-positive ion-ESI–tandem mass spectrometry.

Li et al. (2013) presented an ultrahigh-performance LC-tandem mass spectrometry (UPLC-MS/MS) method for simultaneous quantification of seven plasma biotransformation products of sulfur mustard. Limits of quantification ranged from 0.01 to 5.0 μg/L. The main products found in rat plasma were bis-β-chloroethyl sulfoxide (SMO), TDG, TDG sulfoxide (TDGO), 1,1′-sulfonylbis-[2-*S*-(*N*-acetylcysteinyl) ethane] (SBSNAE), 1,1′-sulfonylbis-[2-(methylsulfinyl)ethane] (SBMSE), and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTESE).

Methods to detect lewisite exposure have been focused on its main biotransformation product, CVAA. Initial methods were developed for environmental samples (Bossle et al., 1989). Methods for CVAA quantification in serum were described by Fowler et al. (1991), as well as Jakubowski et al. (1993). CVAA was derivatized with 1,2-ethanedithiol and quantified using GC-MS. Logan et al. (1996) employed a similar method to detect CVAA in the urine of guinea pigs exposed to lewisite.

52.4.2.7.2 Detection of DNA and protein adducts of vesicants

Adducts of sulfur mustard can be hydrolyzed to release TDG. Lawrence et al. (2008) used this procedure, followed by extraction, derivatization, and GC-negative ion chemical ionization–mass spectrometry. A number of methods have been developed to directly detect and quantify the adducts of sulfur mustard. The most prevalent DNA adduct, *N*7-(2-hydroxyethylthioethyl)-guanine, was directly detected and quantified by Fidder et al. (1994, 1996a), using electrospray LC-tandem MS with multiple reaction monitoring. A more recent work on DNA-adduct quantification was presented by Zubel et al. (2019). Benschop et al. (1997) used an immunoslotblot assay to detect this adduct in blood samples from Iranian patients, in order to verify their exposure that had occurred during the Iran–Iraq War in the 1980s.

Initial efforts by Noort et al. (1996, 1997) to detect the protein adducts of sulfur mustard focused on the 4-(2-hydroxyethylthioethyl)-l-aspartate, 5-(2-hydroxyethylthioethyl)-l-glutamate, cysteine and *N*-terminal valine adduct, and two histidine adducts, *N*1- and *N*3-(2-hydroxyethylthioethyl)-l-histidine. Acidic hydrolysis and pronase digestion were used to release these adducts from globin. Pronase is a mixture of proteinases isolated from the

extracellular fluid of *Streptomyces griseus*. Adducts were derivatized with 9-fluorenylmethyl chloroformate, followed by identification and quantification, using GC-MS.

Even though they constitute only 1%−2% of alkylated amino acids, the *N*-terminal valine adducts were useful for subsequent quantification efforts. *N*-alkylated *N*-terminal valine could be selectively cleaved, using pentafluorophenyl isothiocyanate as a reagent in a modified Edman procedure. The product of this reaction, pentafluorophenyl thiohydantoin, was derivatized with heptafluorobutyric anhydride and quantified by negative ion GC-MS/MS. This method was sensitive enough to confirm an in vivo exposure of guinea pigs 48 h after i.v. administration of 0.5 mg/kg, that is, 6% of the LD_{50} (Fidder et al., 1996b). Recently, an improved method to detect the *N*-terminal valine adduct was presented by Nie et al. (2011) with a lowered limit of detection for identification of the adduct after a 20 nmol/L in vitro sulfur mustard exposure of human blood.

Noort et al. (1999) again used pronase to digest human serum albumin that had been incubated with sulfur mustard. The cleavage product after proteolysis, *S*-[2-[(hydroxyethyl)thio]ethyl]-Cys-Pro-Phe, was analyzed by micro LC-tandem mass spectroscopy. Depending on the protease applied for albumin-adduct enzymatic cleavage different biomarkers can be detected including the above mentioned tripeptide but also the alkylated dipeptide (Cys-Pro) (Gandor et al., 2015; John et al., 2016a,b) or a pepsin-mediated hexapeptide and dodecapeptide (John et al., 2019).

Carol-Visser et al. (2008) developed a method for online pepsin digestion-liquid chromatography-tandem mass spectrometry configuration for rapid analysis of protein adducts of CWAs and demonstrated the detection of specific adducts of sulfur mustard in human serum albumin (along with sarin adducts to human butyryl cholinesterase).

Yeo et al. (2008) presented a verification method for the detection of albumin adducts of sulfur mustard by pronase digestion, followed by LC-MS-MS/MRM analysis. A blood concentration of 50 nM sulfur mustard resulted in formation of adducts that were successfully detected. Adducts of nitrogen mustards could also be detected after exposure to 100 nM HN-2 or 200 nM of HN-1 or HN-3, respectively. A more recent approach made use of proteinase K for proteolysis of the albumin adduct allowing quantification of sulfur and nitrogen mustard adducts (Pantazides et al., 2019).

Incubation of lewisite−protein adducts with BAL is capable of transferring its product 2-CVAA into a BAL-CVAA derivative. This derivative can be quantified using GC-MS. The method is able to detect a 1-nM lewisite exposure of human blood in vitro (Fidder et al., 2000).

A specific β-globin adduct to Cys93 and Cys112 was identified by the use of electrospray tandem mass spectrometry, as well as by chemical transformation with the cysteine-selective reagent vinylpyridine and derivatization by *S*-carbamylation (Fidder et al., 2000). No method for quantification of this adduct was described. As of mid-2008, no specific adducts other than the abovementioned ones have been described in literature.

Despite 90 years of research into the effects of vesicant agents and on medical countermeasures, only very limited data on toxicokinetics are available. However, technological advances in analytical chemistry have contributed to a better understanding of toxicokinetic properties of both sulfur mustard and lewisite, particularly in the last two decades. Knowledge gaps exist, but research efforts continue. The Chemical Weapons Convention (CWC) constitutes enormous progress in efforts to completely rule out future incidents of chemical warfare, and the risk of chemical weapons being used by state parties is probably lower than in any decade since 1910. On the other hand, there is a worldwide asymmetric threat, and the risk of terrorist attacks using chemical weapons cannot be ruled out. Defense experts, including medical chemical defense researchers, will be concerned about these issues for the foreseeable future. It is to be hoped that more comprehensive knowledge on both toxicodynamics and toxicokinetics will contribute to the development of more effective, and possibly causative, therapies of vesicant poisoning. At the same time, the capability to unanimously prove vesicant exposure, even long after the incident, certainly increases the possibility that any use of these CWAs will ultimately be detected and sanctioned. State-of-the-art analytical methods may play an important role in deterring and preventing acts of chemical warfare.

52.5 Concluding remarks and future directions

Measurement of toxicokinetic profiles is primarily motivated to improve the in-depth understanding of the poison's fate in an organism, and thus of pathophysiological consequences. Both are important to optimize antidote treatment and therapy regimens for poisoned humans that intend to boost poison elimination and reverse toxic effects; or at least minimize harm by causal and symptomatic approaches. As outlined in this chapter, the fate of poison is regulated by multifactorial processes characterized by degradation and enzymatic biotransformation, as well as elimination by protein or DNA binding. With respect to state-of-the-art techniques and study design, only a few animal models are being investigated comprehensively for nerve agents, whereas almost no data on sulfur mustard and lewisite are available in the literature.

To make matters worse, almost no human toxicity or toxicokinetic data for CWAs are available due to ethical reasons, hindering specific and tailored medical approaches. Therefore, data are to be obtained from animal studies requiring extrapolation of relevant characteristics to human conditions. As is obvious from the tabular compilations on acute lethal doses of nerve agents in different laboratory animals and different routes of exposure (Table 52.2), especially under consideration of stereoisomeric differences (Table 52.4) and underlying kinetic characteristics in terms of rate constants for serine esterase inhibition and catalytic constants for PTE-mediated hydrolysis (Table 52.3), such data remain incomplete. In addition, interspecies and intraspecies variations are enormous, limiting comparability and transferability of experimental results. There is only a limited amount of data considering specific differences between lethal and sublethal exposure scenarios. Data on tissue distribution in animals are rare and correlation to humans is not clarified conclusively to explain, for example, the larger persistence of nerve agents in the human body. This lack of information indicates future demands of medical defense research. There is also a rising need for combined toxicokinetic and pharmacokinetic data obtained from poisoned organisms under therapeutic treatment. This will help to find the most valuable reactivators of OP-inhibited cholinesterases, which differ dramatically in efficacy in a poison- and species-dependent manner. Taken together, results of future studies should (1) support the development of mathematical models to describe and predict poison and antidote behavior in vivo in humans; (2) unravel novel targets of poison on the molecular or compartmental level, which are useful as biomarkers or to identify additional pathophysiological situations; and (3) establish novel bioanalytical methods and techniques, which allow very low quantification limits and sufficient selectivity for stereoisomers in various tissues and compartments. Whereas a number of studies have been performed using commonly known nerve agents, there remains much more demand for further work with respect to vesicants.

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Chapter 53

Toxicokinetics and toxicodynamics of DFP

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53.1 Introduction

53.1.1 DFP synonyms and scientific publications

Diisopropylfluorophosphate (DFP) is an organophosphorus compound (OP) which is mentioned in the literature using several synonyms (Table 53.1). Soon after its discovery, it was demonstrated to be a very potent cholinesterase inhibitor and toxic compound. However, it has not been used as a pesticide, a chemical warfare agent, or a plasticizer. DFP is commercially available from chemical suppliers and has been extensively used in toxicology and biological research. In April 2014, a search of the PubMed database yielded 3514 results and 4049 in September 2019 looking for all the different synonyms (including the acronym "DFP") in titles and abstracts.

In other searches, using only one of the four MeSH terms (isofluorate, DFP, diisopropylphosphofluoridate, or diisopropylphosphorofluoridate), 3298 results were found. Therefore, the PubMed databases use these four terms as synonyms and the same references are found with each search. They represent most but not all publications involving DFP. A search using the synonyms in (All Fields) gave up to 4726 documents, but the search in (Title and Abstract) was preferred for restricting results to publications in which the compound has a relevant role.

A search updated on September 7, 2019, using all synonyms (including "DFP") and looking at titles and abstracts provided 4049 entries, of which 312 were published after April 2014 (date reported in previous edition of this book). Following manual review, 94 were identified as studies actually using DFP.

It should be noted that with the abbreviation "DFP," many nonrelated publications are found (i.e., "bacterial DFP-flavoproteins"; DFP-10917 a deoxycytidine analog; database fingerprint; deferiprone, a hydroxypyridinone-derived iron chelator; delayed facial palsy; and other). Unfortunately, there is not an established tradition of indicating the CAS number for a unique reference of the chemicals in scientific publications and standard chemical nomenclature is not always used. Therefore, the use of the acronym "DFP" for a publication search gives many irrelevant publications. If the word "DFP" is not included in the search, then results were 2768 detected documents, with 112 published after 2014. Within these 112, 102 had relevant use of the compound for toxicological or biological studies. Combining the initial 94 and the above112 yielded 70 in common and a combined total of 130 new publications from April 2014 to September 7, 2019 (Table 53.2).

Statistics of the distribution of publications over time are shown in Fig. 53.1. The highest rate of publications was during the period 1970−2000. The first publication registered in PubMed was in 1946, while its synthesis was done in the 1930s and the first patent issued in 1938 but published in 1948. Details of the history of DFP are described below.

53.1.2 Research field of the use of DFP

DFP has been used extensively for several reasons:

1. It was one of the first synthesized anticholinesterase OPs. On the basis of a report by Lange (1930), a high yield preparation of DFP was described by Saunders and Stacey (Cambridge, UK) in 1948. A US patent was filed in 1949 and it was proposed as a chemical warfare agent. It was previously synthesized by Schrader (1937) and a patent filed in 1938 but not published until 1951.
2. Since the 1960s, DFP has been commercially available through suppliers of laboratory chemical reagents. DFP with ^{3}H or ^{32}P radioisotopes has been reported in publications since the 1960s (Johnson, 1969a).
3. Other fluorophosphates were synthesized and produced as chemical warfare agents (sarin and soman).

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TABLE 53.1 Identification of DFP and synonyms.

Molecular formula:	C_6-H_{14}-F-O3-P; $(C_3H_7)_2$-P(O)-F
CAS #:	55-91-4
Molecular weight	184.1456
Usual acronym	DFP (a)
Main synonyms reported in ChemIDPlus	Diisopropylfluorophosphate
	Isoflurophate (MeSH, Medical Subject Headings File), (USP, USPDDN, United States Pharmacopeia Dictionary of Drug Names)
Other synonyms in ChemIDPlus	PF-3; Diisopropylphosphorofluoridate; phosphorofluoridic acid, bis(1-methylethyl) ester; isofluorphate; diflupyl; diflurophate; diisopropoxyphosphoryl fluoride; diisopropylphosphofluoridate; *O,O′*-diisopropylphosphoryl fluoride; *O,O*-diisopropylfluorophosphate; fluorophosphoric acid, diisopropyl ester
List of synonyms found in publications referred in PubMed data base	DFP (a); diisopropylfluorophosphatase; diisopropylfluorophosphatases; diisopropylfluorophosphate; diisopropylfluorophosphatetreated; diisopropylphosphorofluoridate; diisopropylphosphorofluoridates; diisopropylphosphorofluoride; diisopropylphosphorofluorofluoridate; diisopropylfluorophosphatase; diisopropylfluorophosphate; diisopropyl fluorophosphates diisopropylphosphorofluoridate; diisopropylfluorophosphate; diisopropylphosphorodiamidate; diisopropylphosphorodiamidofluoridate; diisopropylphosphorodithioato; diisopropylphosphorofluoridate; diisopropylphosphorothioate; diisopropylphosphorothioic; di isopropylfluorophosphonate; di iso propyl fluoro phosphate; isoflurophate
MeSH terms used as synonyms in PubMed	isoflourate; diisopropylfluorophosphate; diisopropylphosphofluoridate;di isopropylphosphorofluoridate

[a] *"DFP" is not completely specific for identification, as it is also used as an acronym for other substances or issues.*
Source: ChemIDPlus in ToxNet databases, US National Library of Medicine (http://toxnet.nlm.nih.gov) and PubMed data base National Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed).

However, DFP is less toxic than the G-series nerve agents and is therefore easier to handle.

4. DFP is an appropriate model compound for toxicological studies and developing prophylaxis and therapeutic strategies and understanding the mechanisms involved in toxicity of OPs. DFP is able to bind to many proteins with serine, tyrosine, and other residues, and therefore is an inhibitor of proteases and other esterases other than cholinesterases. This capacity has triggered interest in using it for toxicological, pharmacological, and biomedical research, as indicated in Table 53.3.

A detailed examination of the 130 articles identified and published during the aforementioned 5-year period produced a tentative distribution of the topics (Table 53.4). More than half (61%) were dedicated to studies related to cholinesterase inhibition in vitro and in vivo, kinetic and molecular mechanisms of inhibition, interaction of OPs, and development and mechanistic studies of reactivators (oximes and others) for therapy and prophylaxis against intoxications of OPs. A large number dealt with in vivo studies as a model of neuroinflammation, status epilepticus, and related therapeutic research (25%). Studies of hydrolysis, biodegradation, and stability were also a significant portion (19%). About 12% dealt with binding to proteases and other proteins, and in some cases, the inhibition by DFP was used as evidence of it being a serine-containing protein. It should be noted that the actual number of papers using DFP for protease inhibition is much higher because when DFP is applied only as a methodological procedure for inhibiting proteases it is not usually mentioned in the abstract or title.

DFP is an inhibitor of the so-called neuropathy target esterase (NTE), and is a critical model compound used to demonstrate the role of aging in the capacity of OP to be an inducer of delayed neuropathy (Clothier and Johnson, 1979a,b) in the usual avian model of OP-induced delayed neuropathy. DFP has been used recently to demonstrate the delayed effects of neuroinflammation in rat (Li et al., 2015) and mouse (Ebrahimi et al., 2018) models.

In brief, there are many studies in which DFP is used either as a model OP compound of toxicity due to binding to esterases (cholinesterases, NTE, and others), as a tool for understanding some biological and toxicological processes, and for developing and studying therapeutic drugs. More than 4000 papers mention DFP, of which 3000 do so in the title or abstract. For 5 years, the individual revision of 130 articles with DFP in the title and abstract indicated that most uses have been for studies related to its properties of cholinesterase inhibition, including kinetic mechanistic studies in vitro and in vivo, with a high proportion using it as a model compound of neuroinflammation and for

TABLE 53.2 Published paper by date publication in searches performed September 7, 2019.

Query	Comment	Items found
Searches in [All Fields]		
Search #1: ((DFP[All Fields]) OR (All synonyms a) [Title/Abstract])	Including "DFP" or All synonyms	5616
Search #2: ((All synonyms a) [AllFields])	Including All synonyms but not the acronym "DFP"	4726
Searches in Title and Abstracts (Including "DFP")		
Search #3: ((DFP[Title/Abstract]) OR (All synonyms a) [Title/Abstract])	September 2019. All documents found with ("DFP" OR (all synonyms))	4049
Search #4: (#1) AND (Date-Publication 2014-04-07 to 2019-09)	Those in search #1 since 2014–19	312
Search #5: Selected 94 relevant (b) documents.	Manually identified reviewing abstracts	94
Searches in Title and Abstracts (Not including "DFP" acronym)		
Search #6: All synonymous [Title/Abstract])	September 2019.	2768
Search #7: (#4) AND (Date-Publication 2014-04-07 to 2019-09)	Since 2014–19	112
Search #8: Selected 106 relevant (b) documents.	Manually identified reviewing abstracts	106
Search #9: (#5) OR (#8)		
(Merging both groups of selected documents)	They represent all the identified relevant (b) papers published since April 2014 to 7 September 2019	130

Indicated results of searches including or not the acronym "DFP," looking in [All Fields] or only in [Title/Abstract] and documents published during 5 years since those reported in the first edition of this book (obtained April 2014 until September 2019).
[a] *"All Synonyms" means the list of synonyms described in Table 53.1*
[b] *Considered "relevant" all the studies actually working or using diisopropylfluorophosphate (DFP) for toxicological or biological studies. Rejected those using "DFP" as an acronym/abbreviation for other substances or other purposes as well as those only using DFP for chemical studies no related with toxicology or biological research.*

simulation neurotoxicity of warfare nerve agents, and also for biodegradation studies and applications to characterization and binding to proteases and other proteins. Some of these aspects are discussed further in the following sections.

53.2 Physicochemical properties and chemical identification of DFP

53.2.1 Chemical structure, identity, and analogy with other nerve agents

DFP, is an O,O′-dialkylfluorophosphate (CAS #55-91-4) synthesized in the 1930s by procedures patented looking either for insecticides, mold control, warfare agents, or fluorine compounds for dental protection. However, it has not been used as a warfare agent. It is classified and labeled in the EU Regulation (CE 1272/2008) following the global harmonized system (GHS) as H300 (*Fatal in contact with skin*) + H310 (*Fatal if swallowed*) + H330 (*Fatal if inhaled*), and with the Precautionary Statements as follows: P260 (*Do not breathe dust/fume/gas/mist/vapors/spray*) and P264 (*Wash hands thoroughly after handling*).

DFP is named and referred to using other synonyms (Table 53.1), although most commonly it is called DFP or diisopropylphosphorofluoridate. Isofluorophate is used as its heading in some databases. Similar to a warfare nerve agent, DFP contains the oxon group (P═O) of the oxophosphates, and therefore does not need the toxic activating reaction needed by the thiophosphates (P═S).

The two organic substitutions with isopropyl groups are bonded to the phosphorus atom through an oxygen atom (alkoxy groups), being a third substitution of the acidic group fluorine (F) which is the most labile group against possible reaction of nucleophilic substitution and considered the "leaving group" or "X-group" (Fig. 53.2). Therefore, DFP may be considered as F-containing oxo-organophosphate, the expression of "phosphate" indicating the two O-substitutions through oxygen atoms.

Other well-known G-nerve agents also contain fluoride (i.e., sarin, soman, and cyclosarin) while tabun contains −CN as a leaving group (Fig. 53.3). However, sarin and soman have one substitution by a P−O bond and another a P−C, so they are usually labeled as phosphonates. Phosphoramidate compounds have also been synthesized with a nonsubstituted amido group (P-NH_2,

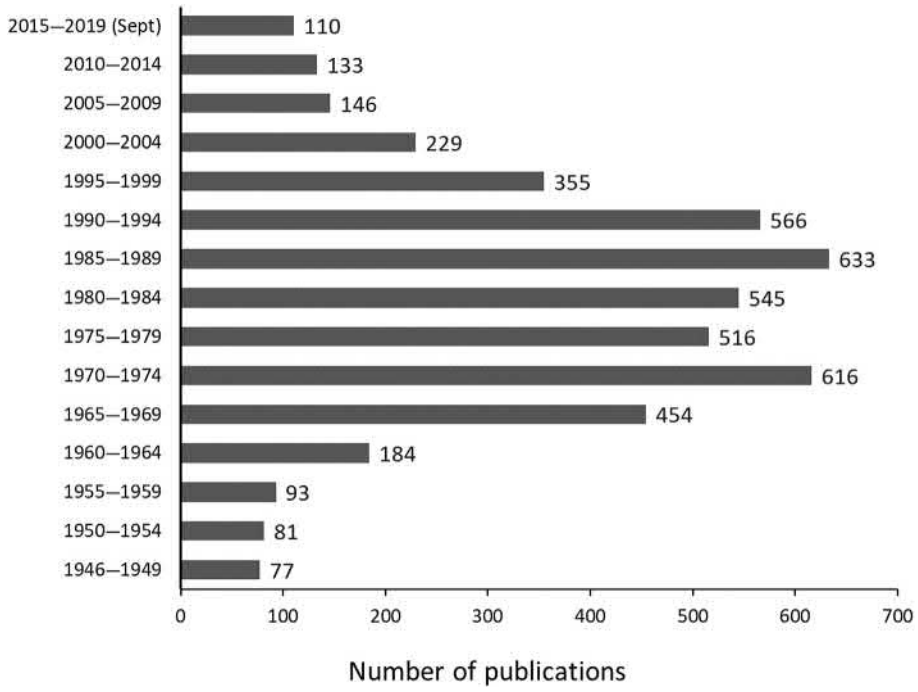

FIGURE 53.1 Evaluation of publications in PubMed containing DFP synonyms in all fields. Data updated September 7th, 2019. Indicated publications in periods of 5 years.

i.e., methamidophos), or mono- (P-NH-R) or disubstituted (P-N-[R_1,R_2]) groups. An example is mipafox (N,N′-diisopropylphosphorodiamidoflouridate).

Mipafox was developed for insecticidal use, but was withdrawn for that application due to its potential for causing delayed neuropathy. It is currently used as a selective inhibitor of NTE (Tormo et al., 1993; Vicedo et al., 1993). DFP has a common property with mipafox for inhibiting NTE (Estevez et al., 2013) and inducing delayed neuropathy.

53.2.2 Physicochemical properties

Table 53.5 shows some basic physicochemical properties of DFP, some nerve agents and mipafox. DFP is a liquid (mp = 82°C) with a clear or slightly yellow color. Dialkyl fluorophosphates was described as producing a pleasant odor by a researcher who inhaled fumes in the process of synthesis and became intoxicated after a few minutes. Melting and boiling points are in the same range as for other nerve agents. The vapor pressure is 0.570 mmHg, which is higher than tabun but lower than sarin. Vapor pressure may be a criterion for assessing the risk of inhalation exposure when handling the pure liquid compound in the laboratory. It is low when compared with other liquids like water (25 mmHg) or organic solvents (i.e., 28 mmHg toluene, 151 mmHg *n*-hexane). Therefore, handling a small amount in the laboratory for experimental studies is viable if the appropriate protective measures are used.

53.3 History of DFP synthesis and its relationship with the development of warfare nerve agents

DFP is used in research to understand the mechanism of toxicity and for studying the therapy and prophylaxis of chemical warfare OP agents due to its relatively low toxicity when compared to the G-series nerve agents [tabun (GA), sarin (GB), soman (GD), or cyclosarin (GF)].

A history of monofluorophosphates has been reported by Peter Meirs (http://www.fluoride-history.de). Inorganic fluorophosphates (sodium monofluorophosphate) and organic aryl or alkyl substitutions were the subjects of filing patents of methods of synthesis looking for substances intended as either insecticides or warfare chemicals.

Willy Lange described the synthesis of some fluorophosphates, difluorophosphoric acid, and patented the synthesis of aryl fluorosulfonates and proposed their use as insecticides (Lange, 1930). It has been described that, in 1932, Gerda von Krueher, one of Lange's students, was involved in fluorophosphates research in laboratory work for her PhD thesis. By heating silver monofluorophosphate with methyl or ethyl iodide, she prepared dialkyl monofluorophosphates. She described that initially the fumes had a pleasant odor but, within a few minutes, she and her colleagues began to suffer pressure in the larynx, difficulty breathing, disturbances of consciousness, blurred vision, and painful sensitivity to light. After several hours, these symptoms resolved. von Krueher described the effects as not being due to the acidic effects but from the small

TABLE 53.3 Type of studies, for which DFP have been used.

Role of DFP and type of studies	Description
Kinetic inhibition and reactivation	Inhibition of cholinesterases in vitro and in vivo, for studying the kinetic and molecular mechanisms of inhibition and interaction of OPs, including development and mechanistic studies of reactivators (oximes and others) for therapy and prophylaxis against intoxications of OPs
Neurotoxicity OP insecticides and warfare agents	Model compound of neurotoxicity and pharmacology related to cholinesterase inhibition. Used as a model OP counterpart similar to chemical warfare G-agents. Model for experimental in vivo studies of neuroinflammation, epilepticus status, and other related clinical status for experimental studying in the development of proposed therapies
Protease inhibitor and phosphorylable proteins	As a tool as irreversible serine of inhibitor in studies in biological processes. Used as evidence that a protease is a serine protease. This is usually done checking also inhibition with PMSF (phenyl methane sulfonyl fluoride)
	Detection of phosphorylable sites in proteins and binding to protein in serine or in tyrosine residues on esterases, proteases, and other proteins
Esterase inhibitor, biological process	In studies related to biological processes when esterases or other DFP-binding proteins were thought to be involved. Coagulation and thrombin and prothrombin inhibition; role of lysozyme; in some cardiovascular process; skin sensitization process; neurodevelopment; neurobehavioral alterations; others
Therapy	In ophthalmological studies related to glaucoma and other disease in which AChE inhibitors have been proposed as therapeutic strategies, i.e.: Alzheimer disease, Parkinson disease, glaucoma
Labeling, cell biology	Use for labeling in study and clinical diagnosis of the turnover and life span of some human cells: erythrocytes, platelets, leukocytes, granulocytes
Delayed neuropathy	In the detection of the target of the organophosphorus-induced neuropathy (OPIDN), and the so-called neuropathy target esterase (NTE) and in the understanding of the molecular mechanism of induction including the role of the so-called "aging" reaction as a mechanism in delayed neuropathy
Other	Other biological and biotechnological applications

No specific statistics were performed for the different types of studies showed in this table. See Table 53.1 for specific statistics based on more recent studies.

TABLE 53.4 Identified published papers during a period of 5 years using DFP for toxicology or biological research.

Classification by issues		Number
1. Model of OP toxicity neurotoxicity related with inhibition of cholinesterases and other esterases		
1.1 Inhibition, kinetic cholinesterases	12 (9%)	
1.2 Oximes, reactivation, protection	8 (6%)	
1.3 Model for in vivo studies of neuroinflammation, status epilepticus, and related therapies	32 (25%)	
1.4 Other neurotoxicity, development and/or in vitro	10 (8%)	
1.5 Intended as model nerve agent (Gulf War illness)	15 (12%)	
1.6 Model of delayed neuropathy, NTE	2 (1.5%)	
Total group 1		79 (61%)
2. OP hydrolysis—biodegradation, stability		25(19%)
3. Proteases, inhibition or characterization of serine-proteases and binding to other serine proteins		15 (12%)
4. Alzheimer disease and other CNS diseases		4 (3%)
5. Other and model of analytical detection		7 (5%)
Total:		130

Based on information obtained from PubMed database of publications since April 2014 (as reported in the last edition of this book) until a systemic bibliographic search on 7 September 2019 (total selected: 130 publications mentioned in Table 53.2).

amount of esters. Other homologs were prepared as di-*n*-propyl and di-*n*-butyl esters. Lange considered that these compounds might be useful for pest control and offered them to the I.G. Farben Industry but the company had no interest at that time. Later, in 1935, at I.G. Farben, Gerhard Schrader, Han Kükenthal, and Otto Bayer (director of research at Farben) patented alkylsulfonates as insecticides (Schrader et al., 1935), claiming that they were more effective than the aryl analogs proposed by Lange in 1930. They also patented dialkylaminophosphorofluorides for insecticide applications (Schrader and Bayer, 1935).

In 1937, Schrader and Kükenthal prepared several compounds and tested them for insecticidal activity. They observed that the most effective were the di-esters with an acidic group of Cl, F, SCN, VNO, and others (Schrader, 1937). The highly toxic compound with the CN group, a *O*-ethyl and a *N*-dimethyl substitution was developed and is now known as tabun. Schrader also described other more effective methods to synthesize esters of fluorophosphoric acid than those described by Lange and Krueger, and synthesized other homologs, among them DFP. A patent was filed in 1938 but kept secret and not published until 1951. This patent does not cover sarin and sarin-type compounds.

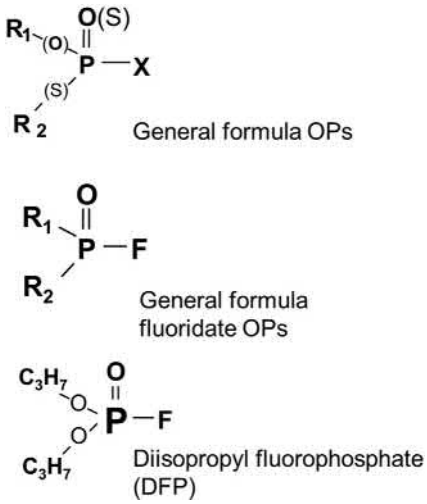

FIGURE 53.2 **General structure of organophosphorus compounds and fluorophosphates.** R is an alkyl or aryl group. X is the so called "leaving group."

Bernard Charles Saunders (UK, University of Cambridge) studied the 1932 report of Lange, prepared several new monofluorophosphoric esters, and tested them for possible use as warfare agents. It was reported to the government in 1941 (Saunders, 1957) and a patent for the synthesis of esters of fluorophosphoric acid was filed in 1943 (McCombie et al., 1944). The method was based on treatment of the corresponding chloro compound with a metallic fluoride such as NaF.

Willy Lange emigrated to the United States, worked for Procter and Gamble, and at the University of Cincinnati. In a work supported by the Ozark Chemical Company in 1943, Lange filed a patent for preparing anhydrous monofluorophosphoric acid and claimed that it would allow it to be used for the synthesis of esters for use as insecticides (Lange and Livingston, 1943). Another related patent was registered in 1944 also claiming the application reacting with alcohol for preparing the corresponding dialkyl esters (Lange, 1946).

In 1944, Hardy and Kosolapoff from the Monsanto Chemical Company patented the production of dialkylfluorophosphates as DFP by a procedure based on the reaction of alcohol with PCl3 producing dialkylphosphite,

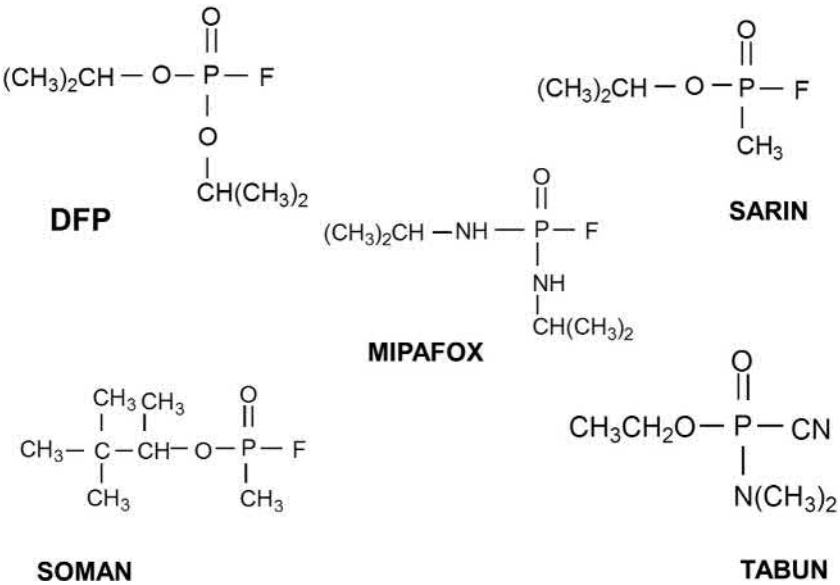

FIGURE 53.3 DFP structure and fluoro and cyano organophosphate analogs.

TABLE 53.5 Some basic physical properties of DFP compared with other nerve agents and F-containing OPs.

Compound	DFP	Soman	Sarin	Tabun	Mipafox
CAS #	(55-91-4)	(96-63-0	(107-44-8)	**(77-81-6)**	(371-86-8)
Molecular formula	$C_6H_{14}FO_3P$	$C_7H_{16}FO_2P$	$C4H_{10}FO_2P$	$C_5H_{11}N_2O_2P$	$C_6H_{16}FN_2OP$
Physical state	Liquid. (oil, clear-yellow liquid)	Liquid	Liquid	Liquid	Solid
MW	184.2	182.2	140.1	162.1	182.2
Melting point	−82.1°C	−42.1°C	−57°C	−50°C	65°C
Boiling point	183°C	198°C	147°C	240°C	125°C (2 mmHg)
	61.9°C (9 mmHg)				
LogP	1.17	1.78	0.3	0.38	0.29
Vapor Pressure (at 20°C)	0.579 mmHg	0.4 mmHg	2.86 mmHg	0.07 mmHg	0.105 mmHg
Solubility water (at 25°C)	15 g/L (very unstable (at pH 7.5, half-life = 1 h); decomposed by alkali)	21 g/L	1103 g/L	98 g/L	80 g/L
Solubility other	Isopropanol: 0.1−0.5 M (stable for months at −70°C)				
Density	1.06 g/mL (25°C)	1.02	1.09		
Recommended temperature	2°C−8°C				

Sources: ChemIDPlusin ToxNet databases, US National Library of Medicine (http://toxnet.nlm.nih.gov); Sigma Chemicals Co, Products information DFP: (http://www.sigmaaldrich.com); Merk Millipore (http://www.merckmillipore.com).

then chlorinating and finally fluorinating with NaF (Hardy and Kosolapoff, 1944).

In 1945, the capacity of inhibiting cholinesterase by DFP was reported by Mackworth (personal communication; Mazur and Bodansky, 1946; Michel and Krop, 1951). The publication of Mazur and Bodansky demonstrated that the inhibition was irreversible and involved binding of the enzyme. DFP and other alkyl fluorophosphate esters were the first compounds reported to be "irreversible" inhibitors. Michel and Krop (1951) reported the quantitative potency of inhibition for cholinesterases (inhibitory properties of DFP on cholinesterases).

In 1947, Lange and Livingstone (Monsanto) published a series of reports about fluorophosphoric acids and their derivatives (Lange and Livingston, 1947). They reported that, "It has recently been shown that the esters of monofluorophosphoric acid, H2P03F, which were previously known to be highly toxic, have a probable usefulness in the treatment of glaucoma and myasthenia gravis, the diisopropyl ester being especially suited for this purpose. Unpublished observations by the senior author also showed that the esters possess insecticidal properties and may act as fumigants" (Lange and Livingston, 1947).

In 1948, a preparation of DFP was described by Saunders and Stacey of Cambridge, UK (Saunders and Stacey, 1948), and a US patent was filed (McCombie et al., 1949). In 1949, another patent was filed for production of mono- and dialkyl fluorophosphates. These were produced by treating alkyl polyphosphates with HF and then named dialkoxy phosphoryl monofluoride (Lange, 1949). The monoesters were purported to have a low toxicity in mammals but did show fungicidal properties. In the same time-period, preparation of DFP by other complicated methods was published, and the effects of DFP on the nervous system and as a potential treatment of human diseases were studied. Further efforts on the application of fluorophosphoric acid and its salts were focused in their application for the prevention of dental disease, that is, as an additive in toothpaste.

B.C. Saunders synthesized DFP and labeled it PF-3. It appears that he based his research on the previous study by Lange and Gerda von Krueger, who synthesized the series of mono-*o*-alkyl fluorophosphoric acid with C1, C2, C3, C4 alkyl groups. However, it was less effective than the G-series agents (sarin, tabun, and soman).

DFP may be synthesized by reactions similar to that used for other OPs. For example, by reacting the alcohol (isopropyl alcohol) with phosphorus trichloride (PCl_3) to form diisopropyl phosphite, and then chlorinating, the chlorine-substituted fluorine was patented by Hardy and Kosolapoff (1944). Therefore, the process of the synthesis of DFP is closely related to the development of the

synthesis of organophosphates for nerve agents, insecticides, and other fluorine compounds for dental application.

DFP has been commercially available since the 1970s from chemical suppliers, such as Sigma. Radiolabeled as [^{3}H]DFP and [^{32}P]DFP it has been used for labeling proteins and for toxicokinetic studies. Therefore, it has been available and used either for understanding its toxicity or for its use as a tool for toxicological or pharmacological research activities.

53.4 Toxicokinetic and biotransformation of DFP and studies on DFPase

53.4.1 Absorption, distribution, and toxicokinetic studies

Radiolabeled [^{3}H]DFP has been used for studying its absorption and biodisposition by inhalation in guinea pigs (Scimeca and Martin, 1988) and mice (Scimeca et al., 1985), and by intravenous administration in mice (Martin, 1985). Skin penetration has been studied in pigs and human skin in vitro (Vallet et al., 2007).

53.4.1.1 Distribution after exposure by inhalation

The tissue disposition of radiolabeled [^{3}H]DFP and its metabolites was studied in guinea pigs after inhalation exposure from 5 min to 24 h after treatment (Scimeca and Martin, 1988). [^{3}H]DFP was rapidly distributed in all tissues. The product of hydrolysis, the metabolite diisopropyl phosphoric acid (DIP), was detected covalently bound to the tissue biphasic curve, with an initial phase representing a very rapid decrease in tissue concentrations, followed by a slower phase of tissue clearance for bound [^{3}H]DFP and free [^{3}H]DIP.

After 4 h, the higher proportion of radioactivity in all the tissues was in the bound form of [^{3}H]DIP. Bound [^{3}H]DIP levels did not follow a biphasic clearance curve and declined at a slower rate than [3H]DFP and free [^{3}H]DIP tissue levels. After 5 min, the greatest accumulation of bound [^{3}H]DIP occurred in the liver (nearly 20% of the total body burden), with a noticeably small amount in the brain (0.1%). Total cholinesterase activity in the brain and red blood cells was inhibited by about 90%, with plasma pseudo- and true cholinesterase activity inhibited by 99% and 97%, respectively.

53.4.1.2 Distribution after intravenous administration

Intravenous administration of [^{3}H]-DFP was studied in mice and the disposition of [3H]DFP in selected tissues and cholinesterase activity and recovery were studied (Martin, 1985). After 1 min [^{3}H]DFP had penetrated the tissues and was irreversibly bound. The tissue concentrations decreased quickly and after 2 h all concentrations were below 50 pg/mg tissue. Most radioactivity was bound to tissue as [^{3}H]DIP but decreased with time in all tissues except liver, kidney, and fat, which reached a maximum at 30 min before declining. Only in the liver and kidney did appreciable quantities of [^{3}H]DIP remain after 3 days.

[^{3}H]DFP was rapidly hydrolyzed to free [^{3}H]DIP, which was found in all tissues within 1 min of [3H]DFP administration. [^{3}H]DIP concentrations were equivalent to or exceeded those of [^{3}H]DFP in all tissues, except the brain.

Cholinesterase inhibition in plasma, diaphragm, and brain following DFP treatment (1 mg/kg, iv) was temporarily correlated with the concentrations of bound [^{3}H]DIP in these same tissues between 1 h and 3 days. Cholinesterase inhibition in the brain and diaphragm did not correlate well with bound [^{3}H]DIP, suggesting there is binding to sites other than cholinesterase.

DFP treatment (1 mg/kg) induced motor hypoactivity up to 6 h after i.v. administration with a time course that did not correlate with free [^{3}H]DFP, bound [^{3}H]DIP concentrations in the brain, or with cholinesterase inhibition in the brain, which suggested that another noncholinesterase interaction was responsible.

Other older studies have been reported on its distribution in oxygenated blood and atrial tissue (Schuh, 1970), excretion in cats (Hansen et al., 1968a), and distribution and metabolism in the guinea pig (Hansen et al., 1968b).

In short, DFP is rapidly distributed, bound to tissues in the form of bound diisopropryl phosphoryl group to proteins, then it is released as a metabolite DIP, and, finally, it is excreted mainly in the urine. There is evidence that a high proportion of binding is to other sites than cholinesterases. Moreover, the time course of CNS effects suggests that other noncholinesterase interactions are involved in DFP neurotoxicity.

53.4.1.3 Skin penetration

Percutaneous penetration of [^{3}H]DFP was tested in vitro with human and pig skin. In the test using intact skin, the absorbed dose after 24 h post-depot was 15.6% ± 1% in pig skin and 9.4% ± 1.5 % in human skin, with a ratio of 1.7 pig/human. Penetration rates between 0.5 and 4 h were 3.22% and 1.63% dose/h, with a ratio of 1.7 for pig/human skin. Similar behavior was observed using split-thickness skin.

The radioactivity quantified in the receptor fluid corresponds to not only intact DFP, but also to its metabolites, both tritiated. In preliminary findings, it was also found that at 24 h post-exposure, more than 99% of DFP reaching the receptor fluid was hydrolyzed into the skin. It was

not specified if this was due to degradation by phosphotriesterases, by binding and dephosphorylation to proteins/esterases, or by spontaneous degradation.

53.4.1.4 Physiologically based pharmacokinetic/pharmacodynamic studies

Two studies with physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) models have been reported (Chen et al., 2009; Gearhart et al., 1990). Gearhart et al. (1990) developed a model for mammals in which the following factors were considered: (1) DFP tissue/blood partition coefficients, (2) rates of DFP hydrolysis by esterases, and (3) DFP-esterase bimolecular inhibition rate constants determined in rat tissue homogenates. Other model parameters were scaled for rats and mice using standard allometric relationships. These DFP-specific parameter values were used to simulate in vivo pharmacokinetic data from mice and rats. DFP concentrations in mouse plasma and brain were successfully simulated with data of a single i.v. injection reported by Martin (1985) as well as AChE inhibition and its reactivation. Effects of repeated, subcutaneous DFP dosing on AChE activity in rat plasma and brain (Michalek et al., 1982; Traina and Serpietri, 1984) were also well simulated, but the return of brain AChE activity to basal levels after cessation of repeated dosing was not well described. The model returned brain AChE activity to the original level, while in the laboratory studies brain AChE never returned to basal levels, even after 35 days. These data suggest modulation of AChE synthesis with prolonged DFP exposure. This study demonstrated the possibility of using a model based on mammalian physiology and biochemistry to simulate in vivo data on DFP pharmacokinetics and AChE inhibition. Scaling of the model between rats and mice was also successful.

Another study developed a model in the mouse and rat (Chen et al., 2009) with the following steps: (1) the influence of the variability of the rate constants for synthesis [K(syn)] and degradation [K(deg)] of AChE, and regeneration [K(reg)] and aging [K(age)] of inhibited AChE on the variability of AChE activity in venous blood and brain was first calculated by a global sensitivity analysis, (2) the mouse PBPK/PD model was calibrated by optimizing the values of K(syn), K(deg), K(reg) and K(age), (3) scale-up of DFP-induced AChE activity was performed from mouse to rat. Validation of the rat model was performed by comparing the time course of venous blood and brain AChE activities from a Monte Carlo analysis to those obtained in vivo. Sensitivity analysis on the verified models showed that K(reg) and K(syn) were the most influential factors of AChE activity at shorter and longer durations, respectively, after DFP challenge, and (4) scale-up of AChE dynamics from mouse to rat successfully evidenced by significant overlapping between the predicted 95th percentile confidence intervals and the experimental data. These approaches hold promise for predictive simulation of organophosphate-mediated AChE inhibition in humans. In vitro toxicokinetic studies for understanding the molecular mechanism of elimination of cyclosarin have been used and DFP applied as a simulant for comparison (Reiter et al., 2014)

53.4.2 Biotransformation of DFP: phosphotriesterases, paraoxonase, DFPPase

DFP is a phosphoric triester and consequently it is not subjected to bioactivation through desulfuration, as in the case of thiophosphates such as chlorpyrifos, malathion, and other OP compounds used as insecticides. DFP can be enzymatically hydrolyzed by the enzymes phosphotriesterases or phosphoric triester hydrolases according to the nomenclature of the International Union of Biochemistry and Molecular Biology (Table 53.6). The hydrolysis of DFP releases the fluoride ion and the acidic moiety of DFP, DIP. This hydrolysis must be considered as a detoxification reaction (Sogorb and Vilanova, 2010).

There are two types of phosphotriesterases, the aryldialkylphosphatase (EC 3.1.8.1) and the diisopropylfluorophosphatase (EC 3.1.8.2). The group EC 3.1.8.1 includes paraoxonases, while the group EC 3.1.8.2 includes the enzymes dialkylfluorophosphatases (also known as DFPases). The enzymes belonging to both families are metal-dependent (calcium in the case of paraoxonases and magnesium in the case of DFPases) and are inhibited by chelating agents (Vilanova and Sogorb, 1999). All these enzymes also contain active centers with discrete binding pockets to accommodate the three ester moieties (Bigley and Raushel, 2013). Although there are mechanistic differences among different phosphotriesterases, in all cases the divalent metal is needed for activation of phosphoryl oxygen that is further attacked by a hydroxide group, causing hydrolysis and the release of the leaving group (fluoride in the case of DFP) (Bigley and Raushel, 2013).

Paraoxonases are expressed mainly in mammals, display a broad range of substrate specificity, and are able to hydrolyze P−F bonds. Consequently, they may also hydrolyze DFP and other analogous warfare agents. DFPases are enzymes with a specific capability to break the P−F bonds and they possess a higher efficacy than paraoxonases for the hydrolysis of DFP and related compounds. However, DFPases are not expressed in mammals. The usual biological source for studying DFPases is the squid (*Loligo vulgaris*) (Wymore et al., 2014; Katsemi et al., 2005; Scharff et al., 2001; Hartleib and Rüterjans, 2001).

DFPases are highly specific for hydrolysis of P−F bonds in DFP. These enzymes can enhance the hydrolysis

TABLE 53.6 DFPase in the IUPAC classifications of enzyme.

3.1 **ESTERASES Hydrolases that act upon ester links**
- 3.1.1 **Carboxylester hydrolases**
  - EC 3.1.1.1 carboxylesterase
  - EC 3.1.1.2 arylesterase...
  - EC 3.1.1.5 lysophospholipase
  - **EC 3.1.1.7 acetylcholinesterase**
  - **EC 3.1.1.8 cholinesterase**...
  - EC 3.1.1.84 cocaine esterase....
  - EC 3.1.1.90
- 3.1.2 Thiolic esters hydrolases
- 3.1.3 Phosphoric monoester hydrolases
- 3.1.4 Phosphoric diester hydrolases
- 3.1.5 Triphosphoric monoester hydrolases
- 3.1.6 Sulfuric ester hydrolases
- 3.1.7 Diphosphoric monoester hydrolases
- 3.1.8 **Phosphoric triester hydrolases (PHOSPHOTRIESTERASES)**
  - **EC 3.1.8.1 aryldialkylphosphatase**
  - **EC 3.1.8.2 diisopropyl-fluorophosphatase (DFPase)**

EC 3.1.8.2. diisopropyl-fluorophosphatase (DFPase)

Reaction: diisopropyl fluorophosphate + H_2O = diisopropyl phosphate + fluoride

Other name(s): DFPase; tabunase; somanase; organophosphorus acid anhydrolase; organophosphate acid anhydrase; OPA anhydrase; diisopropylphosphofluoridase; dialkylfluorophosphatase; diisopropyl phosphorofluoridate hydrolase; isopropylphosphorofluoridase; diisopropylfluorophosphonate dehalogenase

Systematic name: diisopropyl-fluorophosphate fluorohydrolase

Comments: Acts on phosphorus anhydride bonds (such as phosphorus-halide and phosphorus-cyanide) in organophosphorus compounds (including "nerve gases"). Inhibited by chelating agents; requires divalent cations. Related to EC 3.1.8.1 aryldialkylphosphatase. Previously listed as EC 3.8.2.1

Source: Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). In consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN). Enzyme Nomenclature. Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes by the Reactions they Catalyse http://www.chem.qmul.ac.uk/iubmb/enzyme/ (last update 17 February, 2014).

of DFP and analogous warfare agents. As a result, these enzymes can be used either for the deactivation of chemical warfare agents or as prophylactic and therapeutic agents against exposures to these chemicals (Sogorb et al., 2004). However, since DFPases are not expressed in humans, the hydrolysis of DFP by paraoxonases, despite the low specificity, is the only defense against this compound in mammals based on enzymatic hydrolysis. This book contains other chapters dealing with phosphotriesterases (both DFPases and paraoxonases) and readers are referred to those chapters for more detailed information.

Theoretical analysis using the current available computational approaches has been performed to understand the differences among paraoxonase PON1 and DFPase for the preference of substrates (Zhang et al., 2018), as well as specifying technologies for degradation or chemical warfare and the remediation process (Soares et al., 2018).

53.4.2.1 Detoxification of DFP by binding to proteins

In the distribution studies described above, it was observed that after administration of radiolabeled DFP, most radioactivity is bound-DIP and later it is released as free DIP. It is then excreted mainly via the urine. This suggests that, in mammals, binding to a protein by phosphorylation is the main mechanism of withdrawal and detoxification. Catalytically active imine-based covalent binding has been proposed using DFP as a simulant for the detoxification of nerve agents (Royuela et al., 2019).

The mechanism involved in binding to proteins also explains the mechanism involved in toxicity. In Chapter 54, Physiologically based pharmacokinetic modeling of chemical warfare agents, examples of binding to protein are described either as a detoxificating mechanism (i.e., binding to albumin) or binding to the

target esterases causing toxicological consequences (i.e., acetylcholinesterase (AChE) and NTE).

53.4.2.2 The role of albumin in the detoxification of DFP

Albumins exhibit esterase-like activities for carboxylesters (Means and Bender, 1975; Tildon and Ogilvie, 1972; Kokubo et al., 1982). These esterase activities were based in the reversible acylation of a tyrosine residue of the sequence Arg-Tyr-Thr-Arg (position 410 in bovine serum albumin and 411 in human serum albumin) (Peters, 1996a). However, it has also been suggested that this activity, at least in human serum albumin, is the result of the irreversible acetylation of 82 different amino acid residues rather than reversible acylations (Lockridge et al., 2008).

Serum albumin also displays other esterase activities against carbamates, such as carbaryl (Sogorb et al., 2007; 2004) based on the Tyr-411 residue as the main target of the active center. The Tyr was also proposed as a target of phosphorylation of OP compounds, such as phosphoramidates (Sogorb et al., 1998), azamethaphos, chlorfenvinphos, chlorpyrifos−oxon, diazoxon, dichlorvos, malaoxon, pirimiphos-methyl (Carter et al., 2007; Tarhoni et al., 2008), diazoxon, and paraoxon (Sogorb et al., 2008). It is also necessary to add DFP to this list. It is demonstrated that 1 h of in vitro exposure of rat and human serum albumin to 19 μM DFP causes the binding of 0.011 or 0.039 moles DFP/mole of albumin, respectively (Tarhoni et al., 2008). In addition, this binding is prevented by exposure to most of the above-listed OP compounds (Carter et al., 2007; Tarhoni et al., 2008), which suggests that DFP and others are in competition for the same amino acid residue. Other analogous structures to DFP, such as soman, sarin, cyclosarin, and tabun, have been shown to bind to albumin via phosphorylation (Williams et al., 2007; Li et al., 2008; Read et al., 2010).

Mass spectrometry assays have allowed the confirmation of tyrosine-411 as the target amino acid for binding of DFP and analogous nerve agents as well as other OP compounds (Li et al., 2007, 2008). However, other target amino acids for phosphorylation of albumin have also been suggested, specifically four additional Tyr, two Ser (Ding et al., 2008), and several Lys (Grigoryan et al., 2009).

The binding of DFP to albumin, despite the fact it can be considered an enzymatic reaction or a scavenging process with an undetermined number of target amino acids, causes the release of the fluoride ion and generates an adduct between albumin and the diisopropyl moiety. The immediate consequence is that a molecule of DFP is removed from the media and cannot reach the nervous system, where it would cause inhibition of esterases, thereby triggering the neurotoxic effects. Thus, this binding is a detoxification mechanism.

The detoxification of DFP through binding to albumin might seem irrelevant due to its low efficacy in comparison with the detoxification capability of DFPase and paraoxonase. However, the efficacy of this process is based on the high concentration of albumin circulating in the blood (40 mg/mL or 600 μM) (Peters, 1996b), that is, higher than the systemic DFP concentrations compatible with the survival of the individual, and not in a high turnover of the phosphorylated albumin residues. Indeed, the binding of paraoxon to albumin has proved to be responsible for the detoxification of physiologically relevant paraoxon concentrations in mammals (Sogorb et al., 2008). The possibility of applying copper activation of albumin for hydrolysis of OPs has been proposed (Monroy-Noyola et al., 2018).

All the above considerations allow the prediction that the binding of DFP to albumin, in addition to forming adducts with the capability to act as a biomarker of exposure, might be playing a relevant role in the detoxification of DFP, especially considering that paraoxonases are not very efficient in the hydrolysis of OPs with a fluoride leaving group (Sogorb et al., 2004, 2010). DFPases are not expressed in mammals and paraoxonases exhibit a double genetic polymorphism in certain individuals who are more susceptible since they express low amounts of paraoxonase with very low specific activity.

53.5 Acute toxicity of DFP and interaction with AChE

53.5.1 In vitro studies on cholinesterase inhibition

Soon after DFP was discovered, it was demonstrated that it has the capacity to irreversibly inhibit AChE. Mazur and Bodansky (1946) reported in a personal communication that Mackworths had observed inactivation of horse serum cholinesterase by DFP and presented evidence that DFP in very high dilutions *irreversibly inactivates* the cholinesterase of brain, red blood cells, and serum in rabbit, monkey, and man, measuring cholinesterase activity by the liberated CO_2 as a consequence of the acetic acid formation in a bicarbonate−carbonic acid buffer in a classical methodology with Warburg vessels, as had been previously described by Ammon (1930).

Therefore, it was concluded that DFP and the other alkyl fluorophosphate esters were the first compounds reported to be “irreversible” inhibitors of cholinesterases. In contrast with another anticholinesterase drug, physostigmine, withdrawal of the compound by dialysis did not recover the activity.

Michel and Krop (1951) using DFP containing radiophosphorus of high specific activity, demonstrated that the inhibition of cholinesterase by DFP is associated with the binding of DFP phosphorus by the enzyme.

One of the earliest published reports with a systematic study of the inhibitory properties of DFP was that of Koelle and Gilman (1946). This study gave the pharmacological basis of a potential application, as cholinesterase inhibitor, for example, for glaucoma. Comparative inhibition in several species and the differences among brain, red cells, and serum were studied. Differences in the substrate specificity between brain/red cell (so-called "specific") and serum (then called "unspecific") were also noted.

In vitro log pI_{50} was reported to be 6.5 for serum BuChE and 4.2, 4.5, and 4.8 for AChE from rat erythrocyte, brain, and muscle, respectively, with similar values in dogs. In vivo studies after intramuscular injection in rats, dogs, and monkeys were performed and inhibition toxic effects and AChE regenerations was evaluated. Levels in the range of 0.05−1 mg/kg showed significant inhibition of serum ChE and doses of 1−5 mg/kg caused total inhibition of erythrocyte cholinesterase. Human exposure to a dose of 0.5−2 mg/60 kg caused marked inhibition of serum BuChE, without any inhibition of erythrocyte AChE and/or toxic symptoms.

In a study with mipafox, a sarin analog, compared with theoretical studies, it was demonstrated why some compounds, such as mipafox, are refractory to reactivation including when no aging reaction of AChE is occurring (Mangas et al., 2016)

53.5.2 Experimental animal studies on cholinesterase inhibition and acute toxicity

A number of studies have shown AChE inhibition, including gender difference (Table 53.7; Tuovinen et al., 1997) and effects on the different AChE forms in mice, rats, and chickens (Sung and Ruff, 1987; Michalek et al., 1981; Cisson et al., 1981).

Several routes of acute exposure have been tested, including oral, intramuscular, subcutaneous, intravenous, and intraperitoneal. LD_{50} doses are expressed in terms of mg/kg body weight and are shown in Table 53.8.

The main observed effects are those expected from cholinesterase inhibition:

- Respiratory stimulation, cyanosis, and dyspnea;
- Gastrointestinal tract hypermotility, diarrhea, and hypersalivation;
- Behavioral changes, such as tremors, convulsions, seizures, coma, muscle weakness, and alterations in sleep time and righting reflex;
- Alterations in peripheral nerves with neuromuscular blockage and flaccid paralysis, as well as ataxia;
- Fasciculations.

TABLE 53.7 The effect of DFP (4.0 mg/kg) and sarin (0.3 mg/kg) on cholinesterases and CaE activities.

ENZYME control activity nmol/min × mg protein	Compound	Male (%)	Female (%)
Brain AChE (nmol/min × mg protein)			
M:152.0 ± 12.4	DFP	10.2 ± 2.5	10.1 + 0.9
F:145.9 + 10.7	Sarin	10.3 ± 4.5	10.8 + 2.9
Blood AChE (nmol/min × mL)			
1434.9 + 207.5	DFP	48.9 + 9.8	57.0 + 8.5
1485.7 + 46.0	Sarin	55.0 + 8.6	53.9 + 7.2
Plasma BChE (nmol/min × mL)			
3429.9 + 394.6	DFP	3.5 + 0.5	1.7 + 0.1
7115.5 + 728.2*	Sarin	13.8 + 5.4	33.6 + 10.5
Plasma CaE (nmol/minxmL)			
2081.4 + 265.6	DFP	15.2 + 3.1	13.0 + 1.5
2825.6 + 276.0*	Sarin	22.8 + 6.3	25.6 + 2.2

Means +/− SD are given ($n = 5–7$); * $P < .05$ compared to male mice.
Atropine sulfate (37.5 mg/kg) was subcutaneously administered immediately after the organophosphate.
Source: Data obtained from Tuovinen, K., Kaliste-Korhonen, E., Hänninen, O., 1997. Gender differences in activities of mouse esterase and sensitivities to DFP and sarin toxicity. Gen Pharmacol. 29(3), 333−335.

TABLE 53.8 DFP acute toxicity in experimental animals.

Species and route	LD_{50} /LC_{50}	References
Oral		
Rat	5 mg/kg	(1)
	10 mg/Kg	(10)
Mouse	2 mg/kg	(1)
Rabbit	4 mg/kg	(2)
Dog		
Dermal		
Mouse	72 mg/kg	(2)
Rabbit	> 117 mg/kg	(2)
Inhalation		
Rat	360 mg/m^3/10 M	(3)
Mouse	440 mg/m^3/10 M	(4)
Subcutaneous		
Rat	1.44 mg/kg	(5)
Mouse	3 mg/kg	(6)
Dog	3 mg/kg	(2)
Monkey	1 mg/kg	(3)
Rabbit	1 mg/kg	(2)
Intraperitoneal		
Rat	1.28 mg/kg	(7)
Mouse	2.45 mg/kg	(8)
Intravenous		
Mouse	3.2 mg/kg	(9)
Dog	3.43 mg/kg	(2)
Monkey	0.1 mg/kg	(1)
Cat	1.7 mg/kg	(2)
Rabbit	0.3 mg/kg	(1)
Intramuscular		
Rat	1.8 mg/kg	(10)
Rabbit	0.75 mg/kg	(1)
Dog	3 mg/Kg	
Ocular		
Rabbit	1.15 mg/kg	(2)

Data obtained from the database ChemIDPlus: Isoflurophate [USP]US NLM (http://chem.sis.nlm.nih.gov/chemidplus/rn/55-91-4), and referenced as: (1) National Technical Information Service. Vol. PB158–508. (2) Journal of Pharmacology and Experimental Therapeutics. Vol. 87, Pg. 414, 1946. (3) Deutsche Gesundheitswesen. Vol. 15, Pg. 2179, 1960. (4) Nature. Vol. 157, Pg. 287, 1946. (5) Archives Internationales de Pharmacodynamieet de Therapie. Vol. 226, Pg. 302, 1977. (6) Journal of Pharmacy and Pharmacology. Vol. 34, Pg. 603, 1982. (7) Arzneimittel-Forschung. Drug Research. Vol. 14, Pg. 85, 1964. (8) Archivum Immunologiaeet Therapiae Experimentalis. Vol. 23, Pg. 769, 1975. (9) Biochemical Pharmacology. Vol. 15, Pg. 169, 1966. (10) Journal of Clinical Investigation. Vol. 37, Pg. 350, 1958.

Other data of I_{50} for chicken brain AChE are shown in the section of delayed neuropathy later in this chapter for comparing AChE with other esterases.

53.5.3 Studies in man

The acute toxicity of DFP can be well correlated with a rapid and high degree of AChE inhibition. Table 53.9 shows the I_{50} for 45 min reported for human cholinesterases for DFP in comparison with other nerve agents. There is a correlation between AChE inhibition and acute toxicity.

There is very little reported information about accidental acute toxicity in humans. It has been noted that intramuscular and oral doses in the range of 0.07–0.1 mg/kg body weight can produce 50% depression of erythrocyte cholinesterase and moderate clinical symptoms (Grob and Harvey, 1957; Table 53.10).

Other studies of administration of DFP to man in the range of 0.07–0.5 mg/kg were reported with moderate ChE inhibition and some moderate alterations (Grob and Lilienthal, 1947).

53.6 DFP in studies on neurotoxicity and therapy with reactivators

53.6.1 Neuropharmacological studies of the cholinergic system

DFP is used in studies of neurotoxicity and neuropharmacology. In most studies, DFP is used as a model compound causing depression of cholinesterase activity and an increased level of acetylcholine (ACh). Studies include examples of rats surviving status epilepticus induced by DFP (Deshpande et al., 2010), a comparison of strains of rats in their susceptibility to neurotoxicity (Gordon and MacPhail, 1993), age-related differences in the rate recovery of cholinesterase and receptors (Michałek et al., 1990), inhibition of the motor system (Chemnitius et al.,1989), and alterations in energy homeostasis in skeletal muscles (Gupta et al., 1986) and brain (Gupta et al., 2001a,b).

Pharmacological effects and interactions have been the subject of many publications in which DFP is used to induce cholinergic effects and the modulation, agonist, or antagonist of drugs. Examples include: (1) alteration of cannabinoid signaling and its relationship with cholinergic toxicity (Nallapaneni et al., 2008); (2) role of opioid receptors in development of tolerance to DFP toxicity (Tien et al., 2005); (3) preventive effect of galantamine (Saghafi et al., 2013); (4) alteration in the patterns of GFAP upregulation (Liu et al., 2012); (5) alteration of the muscarinic receptor density and their regional adaptation (Yamada et al., 1983a,b), and (6) genetic and sex

TABLE 53.9 Comparison of human cholinesterases inhibition by DFP [I_{50}(45 min)] in comparison with other nerve agents and their LD_{50} for acute toxicity.

Chemical	I_{50} (45 min) (nM) of ChE human				LD_{50} (mg/kg) rats	
	Plasma	Erythrocyte	Brain	Muscle	I.m.	Oral
Sarin	4.2	3	3	4	0.17	0.6
Tabun	13.0	15	15	20	0.80	3.7
DFP	9.5	400	300	250	1.80	6.0

Note: some figures have been rounded.
ChE was measured using acetylcholine as substrate. For the method used, in brain and muscle BuChE activity cannot be discriminated from AChE as no specific inhibitors were used. The I50 (nM) concentration of the inhibitor for a 45 min preincubation is showed.
Source: From Grob, D., Harvey, J.C., 1957. Effects in man of the anticholinesterase compound sarin (isopropyl methyl phosphonofluoridate). J Clin Invest. 37(3), 350–368.

TABLE 53.10 Effect of DFP in humans.

Dose	Effect	References
0.07 mg/kg (i.m.) 0.1 mg/kg (oral)	Reported as dose causing 50% depression RBC ChE in man in vivo	Grob and Harvey (1957)
0.083 mg/kg (i.a.) 0.32 mg/kg (oral)	Reported as dose producing moderate symptoms	Grob and Harvey (1957)
0.48 mg/kg (i.m.) 2.1 (oral)	Estimated lethal dose	Grob and Harvey (1957)
8.2 mg/m^3/10 min	Headache miosis (pupillary constriction): eye	National Technical Information Service. Vol. PB158–508

Source: Data from Grob, D., Harvey, J.C., 1957. Effects in man of the anticholinesterase compound sarin (isopropyl methyl phosphonofluoridate). J Clin Invest. 37(3), 350–368.

differences in the development of tolerance (Russell et al., 1983). The alteration of electrophysiology, nerve conduction, and action potential has been described since the early toxicological studies with DFP (Toman et al., 1947; Couteaux et al., 1946) for which the use of the squid giant axon is a frequent model system (Hoskin and Prusch, 1983).

53.6.2 Neurobehavior and neurodevelopment

DFP has been used as a model compound for studying neurobehavioral alteration related to the increased cholinergic effect caused by AChE inhibition. In some studies, these alterations are compared with those caused by other OPs and have also been related to oxidative stress (Dettbarn et al., 2006; Gupta and Milatovic, 2012; Milatovic et al., 2006, 2014; López-Granero et al., 2013; Gupta and Milatovic, 2014).

Chronic persistent alterations in spatial learning and memory have been described after exposure to DFP (Terry et al., 2012). Zebrafish is also a suggested model for testing developmental effects Faria et al., 2018).

Long-term changes in behavior, such as anxiety, have been reported in adult mice exposed to DFP during the preweanling period (Kofman and Ben-Bashat, 2006). The relationship between behavioral changes and decreased brain cholinesterase activity has been described since the 1950s in studies following intracerebral injections (White, 1956). Since then, many studies have been performed with DFP and other OPs on neurobehavioral effects. Some of the neurobehavioral studies have been related to studies on development by exposure during the prenatal and neonatal periods (Gupta et al., 1985; Das Gupta et al., 1988; Slotkin, 2006; Bushnell and Moser, 2006).

The alteration of circadian patterns (Raslear et al., 1986) and the involvement of nitric oxide in mitotoxicity in muscle and brain hyperactivity induced by DFP have been investigated (Gupta et al., 2001, 2002a; Zaja-Milatovic et al., 2009).

53.6.3 Therapy against anticholinesterase toxicity

Therapy against cholinergic symptoms continues to be a topic of interest for many researchers. DFP has been used extensively as a model compound for inducing cholinesterase inhibition, as well as other nervous system proteins. Studies into the kinetic mechanism of action with oximes and other therapies continue to be an issue of current research. In experimental studies, some of the compounds tested against DFP include bipyridinium oximes (Theirmann et al., 2009), tubocurarine (Barstad, 1956), meptazinol (Galli and Mazri, 1988), 2-PAM (Tuovinen et al., 1996), and quaternary ammonium compounds (Funke et al., 1955).

In silico approaches are introducing new perspectives and capacities for predicting, development, and understanding the mechanism of oxime and other reactivators. For this, DFP has been a relevant tool (Berberich et al., 2016).

53.6.4 DFP in other biological studies

During the 1950s–60s, DFP (isofluorate) was used in ophthalmological studies, including esotropia. Its potential therapeutic application in glaucoma and myasthenia gravis was described in 1947 (Quilliam, 1947) and other studies were published during the 1950s and 1960s in relation to ocular pressure (Zekman and Snydacker, 1953) and retinal detachment (Weekers and Lavergne, 1955).

During the 1960–70s, studies on the mechanism of coagulation were performed using DFP as an agent for blocking endogenous thrombin (Baskova et al., 1970). The interaction of DFP with human blood-coagulating factors has been studied, including factors VII (Osterud et al., 1979), V, and Va (Bartlett et al., 1978), and the Hageman factor (factor XII) (Becker, 1960).

The interaction of the anticholinesterase effect of DFP, with cardiovascular and respiratory reflexes and cardiac failure was also the subject of several studies (Heymans and Pannier, 1946; Wolthuis and Meeter, 1968). During the 1970s, a methodology was developed and applied based on labeling proteins with [^{32}P]DFP for studying the turnover, life-span, and survival of human blood cells (Matsuda, 1969). These approaches have also been applied to studying the kinetics of platelets (Ginnsburg and Aster,1973; Ebbe et al., 1966, 1970; Leeksma, 1963), the survival of erythrocytes (Derelanko, 1987; Cline and Berlin, 1963: Hjort and Paputchis, 1960; Saito and Sakai, 1977; Manunta and Cancedda, 1975), and the kinetics of granulocytes and neutrophils (Vorob'eva, 1976; Nowotny et al., 1978; Dresch et al., 1971).

53.7 Interaction of DFP with other esterases

53.7.1 Serine proteases and albumin: role of tyrosine residues

Serine proteases are proteins containing a serine residue in their active catalytic center acting in a molecular mechanism similar to esterases. Therefore, they are susceptible to phosphorylation by OPs in general and DFP in particular. This has been the basis for DFP use in biochemical studies for blocking serine-containing esterases, and thereby stabilizing proteins and avoiding proteolysis in tissue homogenates. Moreover, DFP has been used as a tool for specific studies either for understanding the mode of action of proteases or for biological studies, for testing the role of proteases in some biological processes. DFP-inhibited chymotrypsin and chymotrypsinogen were studied using P^{31} NMR (Reeck et al., 1977). DFP induced inactivation of bovine trypsinogen and chymotrypsinogen (Morgan et al., 1972). The subtilisin serine protease has been analyzed by NMR (Chen et al., 2008). The involvement of DFP-sensitive proteasomes in the control of oocyte maturation has been evaluated (Takahashi et al., 1994). The evidence of the role of tryptophan residue in the interaction of DFP with alpha-chymotrypsin has been reported (Wooton and Hess, 1960). The effects on sulfhydryl proteases have also been demonstrated (Heinicke and Mori, 1959).

Human serum albumin has been demonstrated to show arylacylamidase activity that is sensitive to DFP (Manoharan and Boopathy, 2006). A tyrosine residue has been characterized as a target of the binding of DFP to albumin (Means and Wu, 1979). Evidence has been reported that the tyrosine residue 411 is responsible for *p*-nitrophenyl acetate catalytic hydrolysis (Tildon and Ogilvie, 1972) and that it has a critical role in the detoxification of OPs such as paraoxon and diazoxon, and carbamates such as carbaryl (Sogorb et al., 1998, 2004, 2007, 2008). Therefore, albumin has been suggested as a biomarker of OP nerve agent exposure (Read et al., 2010; Lockridge et al., 2008).

53.7.2 Inhibition of soluble PVases of peripheral nerve by DFP

In the peripheral nerves, soluble carboxylesterases measured with the substrate phenylvalerate (PV) were found to have a high sensitivity to DFP. Tissue preparations were preincubated with DFP at nanomolar concentrations (up to 10 nM) for different inhibition times (up to 120 min; Fig. 53.4) and with DFP at nanomolar concentrations (up to 1000 nM) for 30 min of fixed inhibition time (Fig. 53.5). Inhibition data were analyzed with model

equations of one, two, and three sensitive (exponential) components, with or without resistant components. The best model according to an F test in both experiments was a model composed of two sensitive enzymatic components and one resistant to DFP. Around 84% of PVase activity was highly sensitive to DFP with I_{50} (30 min) of 0.8−1.2 nM and around 16% was resistant (Table 53.11). The number of enzymatic components and proportions are similar to the results obtained in inhibition experiments with mipafox, PMSF, paraoxon, and S9B (Estévez et al., 2004, 2010, 2011, 2012).

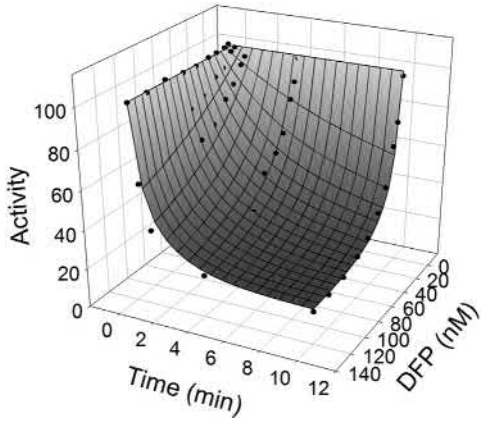

FIGURE 53.4 Inhibition kinetic of esterases by DFP. Example of a 3D representation of the inhibition kinetic of soluble peripheral nerve phenyl valerate esterase activity (PVase) by DFP. Inhibitory surface obtained by fitting the 3D model equation to the data corresponding to DFP inhibition. The surface reflects the result of the best model according to the F test. It corresponds to a model with two sensitive enzymatic components plus other resistant.

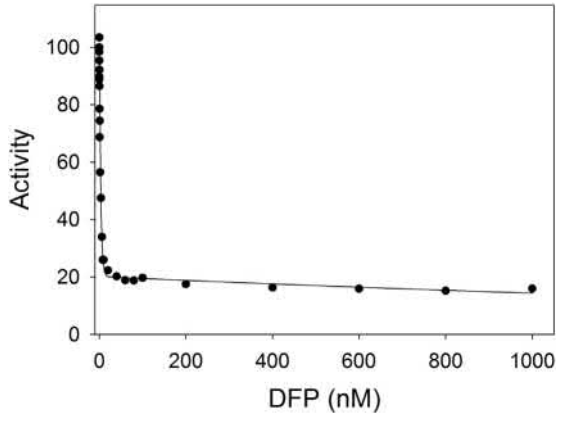

FIGURE 53.5 Fixed time inhibition. DFP 30-min fixed-time inhibition curve of soluble peripheral nerve phenyl valerate esterase activity (PVase). The curve was fitted to the best model according to the F test (two exponential and a resistant component). Each point represents the mean of three replicates (SD < 5%).

53.7.3 DFP- and OP-induced delayed neuropathy and neuropathy target esterase

53.7.3.1 Phosphorylation site identified by radiolabeled DFP

Organophosphorus-induced neuropathy is a neurodegenerative syndrome induced by some OPs (Johnson, 1982). An epidemiological outbreak was first described as a consequence of an adulterated alcoholic drink during the US Prohibition (Smith and Elvove,1930; Smith et al.,1930) and an episode in Morocco in 1959 by consumption of a lubricant oil (Travers, 1962) for which tri-*o*-cresyl phosphate was identified as the causative agent. It was demonstrated that neither AChE nor BuChE inhibition were involved as a mechanism for inducing this syndrome (Aldridge and Barnes,1961). Adult hens were shown to be an appropriate animal model for testing this syndrome in a toxicological effect protocol. The search of the target biomolecules triggering the mechanism was approached using radiolabeled [^{32}P]DFP (Johnson,1969a). Brain tissue treated with [^{32}P]DFP indicated binding of DFP to all sensitive phosphorylable sites in proteins. The fraction of sites that can be protected by a previous incubation with a non-neuropathic OP (TEPP) were considered irrelevant sites. From the remaining sites, the small number that can be protected by binding with a neuropathic compound (mipafox) were considered candidates to contain the target of the toxic mechanism. From the total phosphorylable sites (around 600 pmol/g fresh tissue), about 200 pmol/g was blocked by the non-neuropathic TEPP. The fraction of phosphorylable sites is a potential target for binding with mipafox and was quantified to be about 33−39 pmol/g (Johnson, 1969a).

TABLE 53.11 Inhibition by DFP on esterases component in soluble fraction of peripheral nerve.

	*E*1 (%)	*k*1 ($nM^{-1}.min^{-1}$)	I_{50} (30 min) (nM)	*E*2 (%)	*k*2 ($nM^{-1}.min^{-1}$)	I_{50} (30 min) (nM)	*R* (%)
A	38.9	0.0187	1.2	43.3	$4.1 \cdot 10^{-3}$	5.7	17.9
B	40.7	0.0274	0.8	41.7	$5.2 \cdot 10^{-3}$	4.4	17.6

The kinetic constants (ki) and the proportions of obtained esterase components from the different inhibition experiments with DFP. The I_{50} values were calculated from the kinetic constants for each component. (A) Experiment of time-progressive inhibition with different concentrations in a 3D fitting; (B) Experiment of fixed time inhibition with different concentrations. The R^2 coefficients were: 0.980 (A); 0.986 (B).

53.7.3.2 The target site identified as an esterase: neuropathy target esterase

Johnson (1969b) assumed that phosphorylable sites would be serine-containing proteins. Several carboxylesters were tested looking for a selective substrate able to interact in the same site (able to reduce the speed of [^{32}P]DFP labeling on the mipafox binding fraction). Phenyl-phenyl acetate (PPA) was selected as that substrate. In other studies, phenyl valerate (PV) was observed to be more selective and has been used for decades for testing the target site, the neurotoxic esterase, and was later named the NTE. NTE has been monitored as the PVase activity resistant to 40 μM paraoxon (20 min) ("B" activity) and sensitive to 40 μM paraoxon plus 60–150 μM mipafox ("C" activity) with NTE being the difference between the activity in B and C.

53.7.3.3 Protection and induction of neuropathy: the role of the aging reaction

It has been noted that some carbamates or esterase inhibitors, if dosed before DFP, were able to protect the onset of neuropathy (Johnson and Lauwerys, 1969). A series of studies were conducted for understanding the age-related sensitivity, structure–activity relationship with many OPs (Johnson, 1975a), motor nerve dysfunction (Lowndes et al., 1974), and protection by carbamates, phosphinates, and sulfonyl fluorides (Johnson, 1975b).

While some OPs include neuropathy, phosphinates, carbamates, and sulfonyl fluorides do not, but they rather protect from a further dose of a neuropathic compound. It was hypothesized to be related to the ability of the OP of the dealkylating reaction called an aging reaction. This was more clearly demonstrated by using radiolabeled DFP (Clothier and Johnson, 1979a,b).

The aging process involves dealkylation of one isopropyl group (Fig. 53.6). This was demonstrated by both molecular and enzymatic approaches. The loss of time of the capacity of the inhibited NTE to be reactivated by a nucleophilic reagent (fluoride ion) was demonstrated confirming the aging reaction. In contrast with AChE, the dealkylated isopropyl group in the DFP-inhibited NTE remains bound to the other site ("Z") of the protein (Fig. 53.7). This group may be released to the medium as isopropyl alcohol by alkaline treatment. This was demonstrated molecularly using DFP labeled with ^{3}H and ^{32}P. The molar ratio (^{3}H-isopropyl)/(^{32}P) is 2:1 in the inhibited enzyme and it is converted to 1:1 in the aged protein, confirming that one of the isopropyl groups has been released.

The structure–activity relationship, the relationship of the capacity to induce neuropathy, and the capacity of causing the aging reaction have been demonstrated in a wide range of OPs (Johnson, 1982). Therefore, the proposed hypothesis was that blocking the site itself is not a sufficient requirement and that an additional modification of the function of NTE is needed. The protein alteration after the aging reaction is able to trigger the pathophysiological process to induce neuropathy.

This pathological process is involved in a number of molecular events, including alteration of neurofilament phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase. Alteration of the microtubule has been reported from the laboratories of Abou-Donia and Gupta (Gupta and Abou-Donia, 1993, 1999; Gupta et al., 2000). Also, using DFP as a model, c-fos mRNA expression has been reported (Gupta et al., 2002b; Damodaran and Abou-Donia, 2000; Damodaran et al., 2000, 2001), as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the CNS of hens treated with DFP (Damodaran et al., 2002). Early alterations are induced by DFP on the PKA/p-CREB pathway and differential persistence of beta-tubulin subtypes in the CNS of hens are proposed to contribute to OPIDN (Damodaran et al., 2009).

53.7.3.4 Testing delayed neuropathy

The capacity of inducing neuropathy means that the compound should be able to induce a high degree of inhibition of NTE in a condition in which the animals survive the

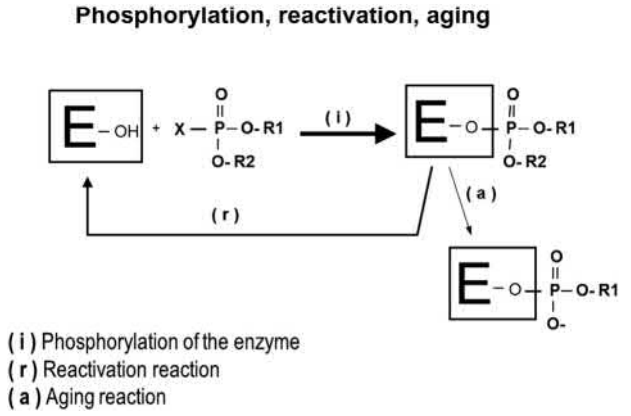

FIGURE 53.6 Reaction of phosphorylation and aging of OPs on esterases. R is an alkyl or aryl group. X is the so called "leaving group."

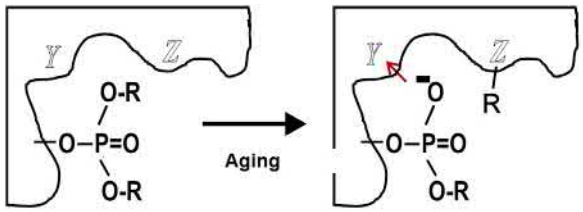

FIGURE 53.7 Aging of the DFP phosphorylated NTE protein. The aging process is a dealkylation reaction. In the case of AChE, the alkyl group is released to the medium as the corresponding alcohol. This figure represents the case of NTE with DFP in which the isopropyl group remains attached to the protein and is released to the medium only after an alkaline treatment. The nature of the "Z" site remains unknown.

acute cholinergic effect. A standard test has been adapted by the OECD as protocols 418 and 419 (OECD, 1995a,b) and by other regulatory agencies. The method involves dosing of animals with a single $5 \times LD_{50}$ dose or repetitive dose in animals protected with atropine and observed for 21 days, accompanied with measuring NTE activity in the nervous system in a group of animals sacrificed 24 h after dosing.

An in vitro approach has also been proposed based on the relative inhibition of AChE and NTE in the hen brain homogenate or in human neuroblastoma cells, followed by testing the aging reaction by the loss of capacity or being reactivated with a nucleophilic reagent (Sogorb et al., 2010). Examples of the ratio of I_{50} AChE/NTE for some nerve agents are shown in Table 53.12. The higher ratio suggests a higher possibility of inducing delayed neuropathy.

Delayed neuropathy has been tested in adult hens/chickens, which are considered the only suitable model. However, repeated exposure with DFP has demonstrated a structural disruption of myelinated axons and persistent impairments of axonal transport in the brains of rats (Naughton et al., 2018). This and other studies in rodents suggest that the rodent model might also be suitable for the neurodegenerative effects caused by OPs. To date, only hens have been considered a suitable model of delayed neuropathy caused by DFP and other OPs after an acute dosing.

Advanced molecular approaches are still needed to be applied to DFP studies. Proteome analysis of rat plasma after DFP intoxication have been evaluated as a simulant of nerve agents (Chaubey et al., 2019).

53.7.3.5 Molecular and genomic characterization of NTE and its role in embryonic development

The molecular identification of NTE protein and its genomic identification (encoding gene: PNPL6) was the result of research conducted by Glynn et al. (1993). For this process, proteolysis of [^{3}H]DFP-labeled polypeptide was also approached. After isolation, using a specific biotinylated inhibitor, molecular cloning was possible (Glynn et al., 1994). The protein catalytic domain was isolated and used for some studies and phospholipase activity was demonstrated and proposed to be involved in neuronal maintenance (Atkins et al., 2002; Glynn, 2005; Mühlig-Versen et al., 2005; Read et al., 2009).

A role of NTE in the embryonic development was envisaged in studies with NTE-deficient mice (Moser et al., 2004). The expression of NTE in mouse embryonic stem cells in differentiation (Pamies et al., 2010) and the alteration of pathways during differentiation by silencing mRNA NTE encoding gene (PNPL6) in mouse and human cells (Pamies et al., 2014a,b), but not inhibition by a neuropathic compound such as mipafox (Pamies et al., 2014a,b). This suggested that its role in embryonic development has a relationship neither with its enzymatic property nor with the same mechanism inducing delayed neuropathy in adults.

In short, the history of understanding delayed neuropathy, the identification of the target protein and the molecular events including the aging reaction in the mechanism inducing the neurodegenerative syndrome, has been very closely related to the use of DFP as a tool for research studies.

TABLE 53.12 I_{50} on AChE and NTE.

	NTE I_{50} (uM)	AChE I_{50} (nM)	AChE/NTE
DFP	0.930	1050	1.13
Sarin	0.338	1.9	0.0056
Soman	0.377	0.46	0.0012
Tabun	6.650	3.5	0.00053
VX	250.000	0.36	0.0000014

Source: From Gordon, J.J., Inns, R.H., Johnson, M.K., Leadbeater, L., Maidment, M.P., Upshall, D.G., Cooper, G.H., Rickard, R.L., 1983. The delayed neuropathic effects of nerve agents and some other organophosphorus compounds. Arch Toxicol. 52(2), 71–82.

53.8 Concluding remarks and future directions

DFP is a dialkylfluorophosphate synthesized in the 1930s when looking for insecticides, warfare agents, and other applications. Although it has never been used as a chemical warfare agent, it has been extensively used in research as a model compound of AChE inhibition and in other toxicological, pharmacological, and biomedical studies, due to its lower toxicity compared to the G-series nerve agents. DFP is detoxified by hydrolysis by A-esterases as the enzyme DFPase (not present in human) and by binding to B-type esterases as the tyrosine residue of albumin and the serine residue on cholinesterases. The acute toxicity is caused by inhibition of AChE and delayed neuropathy is related to the initial inhibition of the so-called NTE. DFP is known to bind and inhibit serine-proteases and other unidentified esterases. DFP is still used as a tool for many toxicological and pharmacological applications in which the consequence of protease inhibition or cholinergic effects is needed for studying a pharmacological or cell biology process.

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ChemIDPlusin ToxNet databases, US National Library of Medicine. <http://toxnet.nlm.nih.gov>.

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Chapter 54

Physiologically based pharmacokinetic modeling of chemical warfare agents

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54.1 Introduction

Pharmacokinetics (PK) is the quantitative determination of drug or chemical movement (time-course concentration) throughout the body. One of the basic principles of pharmacology and toxicology is that the effect of a drug or chemical is directly related to its concentration at some target site or receptor in the different tissues of the body. This premise is the critical reason and continued justification for the development of improved PK descriptions of chemical warfare agents (CWAs). Mathematical models are applied to this type of data to simplify or reduce the description of the basic chemical–biological processes of absorption, distribution, metabolism, and excretion (ADME). These model types are driven by the basic premise that significant pharmacological or toxicological understandings are gained by knowing the internal drug/chemical concentration at the target site. Most often termed "classical" PK models, they reduce the entire body mass to either one or two mathematical compartments that represent the volume of blood, plasma and/or readily accessible extracellular spaces, and a deep compartment storage representing other tissues. The advantage of this analytical approach is the determination of a simple global description of a chemical's behavior in the body, and potentially the concentration at major target receptor sites. The major disadvantage is that these empirical models are poor at interspecies extrapolation, since the parameters do not have a physiological interpretation, and it is difficult to predict how they change when the underlying physiology changes.

Physiologically based pharmacokinetic (PBPK) models are a special type of PK model that attempts to provide more definition to the model analysis by incorporating physiological factors into the model design, like tissue volumes, blood flow rates, and species-specific enzyme characteristics that can more accurately differentiate the dose–response relationship for a chemical or drug in one species from that of another species. The power of this approach is to be able to perform laboratory studies, both in vitro and in vivo, in common experimental species to develop a complete PBPK model, and then extrapolate these results to predictions in humans based on in silico experimentation.

PBPK models have become useful analytical tools to interpret PK data and to interpret data from complex chemical exposure scenarios. These models are mathematical constructs that allow the coordination of species-specific physiology, chemical-specific information, and the experimental protocol for the chemical(s) of concern. The power of PBPK models lie in aiding the ability of scientists and decision makers to simulate the time-course concentration of chemicals in experimental animals and humans and to better determine estimates of actual chemical doses delivered to the target tissue, thereby providing a better prediction of response. Due to the physiologically based nature of these models, simulations of experimental data can be performed by one exposure route to validate the PBPK model, and then this model can be used to simulate and predict the kinetics and pharmacodynamics (PD) in the human by one or multiple exposure routes. This provides decision makers with a fairly rapid method of comparing

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results from in vitro and in vivo laboratory studies, to potentially real-world exposure scenarios. Perhaps the most useful application of these models lies in a strongly animal data-based PBPK-PD model being used to make predictions about human responses to CWAs, across a broad range of doses and CWA types, in light of not being able to actually conduct human exposure studies.

CWAs are represented by any one of a number of chemicals exhibiting very high toxicity by various mechanisms. This chapter covers CWAs with structures as simple as carbon monoxide (CO) and as complex as botulinum toxin or ricin proteins. While this chapter could address the development of PBPK models of CWAs in general, the focus will primarily be on the organophosphate based nerve agents typically represented by sarin (GB; isopropyl methylfluorophosphonate).

54.2 Development of PBPK models

Most attempts at describing PK and PD descriptions of CWAs have used classical kinetic models that are often fit to one set of animal experimental data, at lethal doses, with extrapolation to low-dose or repeated-exposure scenarios with limited confidence. This is due to the inherent nonlinearity in high dose to low-dose extrapolations. Also, the classical approach is less adept at addressing multidose and multiroute exposure scenarios, as occurs with agents like *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX), where there is pulmonary absorption of agent as well as dermal absorption. PBPK models of chemical warfare nerve agents (CWNAs) provide an analytical approach to address many of these limitations.

There are only a few PBPK models that have been developed to describe the PK and PD of CWNAs. Maxwell et al. (1988) developed a PD model for soman in the rat describing the inhibition of acetylcholinesterase (AChE) in different tissues, with mass balance equations including parameters for blood flow, tissue volumes, soman metabolism and tissue/plasma partition coefficients. This effort resulted in an accurate prediction of AChE activity in eight different tissues after intramuscular soman dosing and was able to reproduce dose–response inhibition from 10% up to 100% of the brain AChE activity. Gearhart et al. (1990) used diisopropylfluorophosphate (DFP) as a model compound for CWNAs, to develop a PBPK-PD model describing the PK of DFP and the inhibition of AChE and BuChE in all the pertinent tissues of the body (Fig. 54.1). This model construct was able to predict the PK of DFP and inhibition of AChE and BuChE after acute and repeated doses by three different routes. Recently, the PBPK-PD model for DFP was reparameterized to predict GB kinetics, inhibition of AChE, and regenerated GB from bound AChE sites on red blood cells (RBCs; Gearhart et al., 2005).

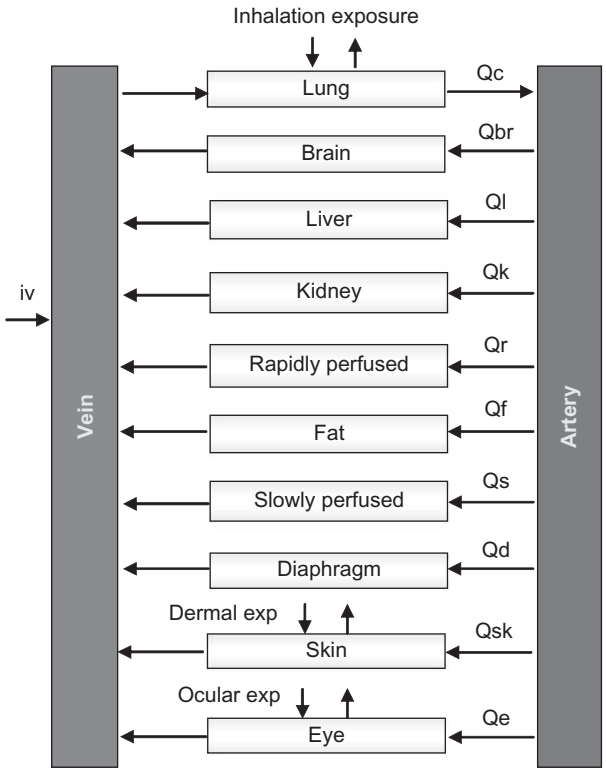

FIGURE 54.1 PBPK-PD model schematic of sarin in the Hartley guinea pig. This model structure allows for the simulation of experimental studies with dosing by IV or SC dosing and IH exposure. This model design was based on Gearhart et al. (1990) and adapted to simulate the PK and PD of sarin in the guinea pig.

The most recently developed PBPK-PD models for CWNAs have continued to focus exclusively on the most potent G agent, soman (GD), due to its rapid aging rate for permanent binding to serine sites on cholinesterase and resultant resistance to therapeutic countermeasures. The main expanded application in these modeling efforts is to multiple species, providing greater confidence in the application of the PBPK-PD method for eventual extrapolation to predictions in the species of concern (namely, humans). Sweeney et al. (2006) augmented the intravenous (IV) exposure model of Langenberg et al. (1997) for GD in guinea pigs, to predict subcutaneous dosing and inhalation exposure, as well as adding the rat and marmoset species. Both of these changes are significant additions, as subcutaneous dosing is the most common experimental method for controlled CWNA challenge to determine efficacy of therapeutic treatments. Inhalation is the primary route of threat exposure for G agents, which tend to readily form vapors, and any of the other CWNAs which, while having very low vapor pressures, still can present an inhalation hazard via aerosol droplets.

Chen and Seng (2012) extended the PBPK model simulation for GD from these previous efforts to include the

pig species, as well as the PD component predicting the inhibition of AChE and carboxylesterase (CarbE) in blood and tissues. One significant area of needed validation from Chen and Seng's model is the assumption that tissue/blood partition coefficients for GD were identical across all species for all tissues, as well as other model parameter assumptions, which could have a critical impact on the broader application to more diverse simulation scenarios (Ruark et al., 2013). For the most significant step of extrapolation to the human species and making predictions about CWNA lethality, few of these parameter values are likely to be available, an area in which Quantitative Structure Activity Relationship (QSAR) analysis can provide a viable impact (Knaak et al., 2004; Ruark et al., 2013).

There are three basic critical components to PBPK models: (1) species-specific physiological parameters, (2) chemical-specific parameters, and (3) specific experimental details for the studies to be simulated. Species-specific physiological parameters are the organ weights and blood flow rates for the defined compartments in the PBPK model. These values are most often available in the published literature and when lacking, are derived from the closest like species. Chemical-specific parameters that are unique for each chemical are the tissue solubility (partition coefficient), metabolism of the parent compound, and plasma and tissue-binding characteristics. Tissue solubility is most often measured experimentally for the volatile CWNAs by the vial equilibration method (Gargas et al., 1989). Existing models for the physiological distribution of CWNAs are based on a limited number of data sets for each agent and typically rely on single exposures at relatively high doses, often in the supra-lethal range (van der Schans et al., 2003). Mathematical models of these data have been developed, but extrapolation to low-level exposures has proven problematic (Sweeney and Maxwell, 1999). Further, there are very little experimental data and essentially no modeling efforts for repeated low-level exposures (Benschop et al., 1991). Thus, new experimental data at the appropriate dose ranges appear to be required for further refinement and extension of existing PBPK models.

Much of the animal data on the biological effects of low-level exposure to GB and other nerve agents has been collected via the subcutaneous or intramuscular route, particularly in guinea pigs, because of their lack of CarbE. A good deal of data has also been collected by this dosing route or intramuscular dosing in rats, miniature pigs, and nonhuman primates. The primary exposure route of interest for human exposure is almost exclusively inhalation or dermal contact, so the quantitative implications of these laboratory exposure data for human health risk assessment is not straightforward. The most reliable tool for extrapolating across both species and exposure routes is PBPK modeling, in which species differences in physiology are taken directly into account, and equivalent systemic and tissue doses for different routes of exposure can be calculated. Such extrapolation procedures, exemplifying the integration of diverse data sets in both animals and humans, are illustrated in Fig. 54.2. Here, the lower pentagon represents the dermal (or subcutaneous) exposure route for different species, while the upper pentagon represents inhalation exposure. The arrow represents the extrapolation of subcutaneous exposure data in the guinea pig to apply them to human inhalation exposure situations. In this example, the relevant biological effects observed in the guinea pig at specific subcutaneous exposures are noted. The guinea pig PBPK model then allows blood and tissue concentration time-courses corresponding to these doses to be calculated. Assuming that similar biological effects occur at similar target-site concentrations across species, the necessary inhalation exposure corresponding to the same blood and tissue concentrations in the humans can then be back-calculated using the human PBPK model for the agent. By using PBPK models in this way, human inhalation exposures are thereby quantitatively linked to the rich animal response and health effects databases.

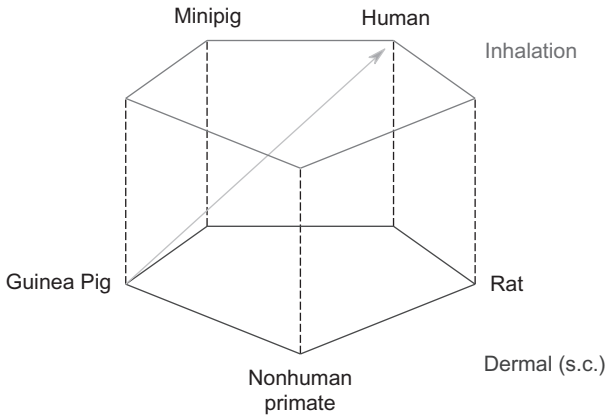

FIGURE 54.2 Schematic illustration of combined interspecies and route-to-route extrapolation. The arrow represents the extrapolation of guinea pig subcutaneous exposure data to human inhalation exposure situations.

54.3 Need for improved measures of CWNA exposure—the use of PBPK analysis of data

Historically, cholinesterase activity has been used to evaluate exposure of humans and animals to nerve agents. Unfortunately, circulating cholinesterase activity is a relatively insensitive and imprecise marker of nerve agent exposure. By contrast, regeneration of nerve agents from inhibited enzymes using a high fluoride concentration

(fluoride regeneration) is well suited to monitoring absorbed doses resulting from low-level exposures to GB, GD, and other CWNAs (Polhuijs et al., 1997; Jakubowski et al., 2001, 2003; Adams et al., 2004). This technique has not yet been applied systematically for kinetic modeling of CWNA exposures in guinea pigs. Recently, a method for regenerating GB from blood and tissue has been developed and validated in rat, guinea pig, and pig models and provides a sensitive and quantitative means for estimating the kinetics of GB in blood (Jakubowski et al., 2004). This method is based on fluoride ion regeneration of the protein-bound agent. Fluoride ion-regenerated sarin (R-GB) was found in blood and tissues of miniature pigs exposed to sarin vapor levels ranging from miosis to lethality. The R-GB in these samples was analyzed by gas chromatography−mass spectrometry (GC−MS) after a C18 solid-phase extraction sample preparation that included fluoride ion addition and pH adjustment. Serial blood samples taken before and during miosis-level GB inhalation exposures resulted in RBC R-GB levels that steadily rose during the exposure. This demonstrated the ability of the R-GB assay as a biometric of exposure. The slopes of the lines created by plotting R-GB versus time closely correlated to the experimental exposure level. Therefore, the rates of GB absorption in these animals were proportional to the GB exposure concentration. In contrast to R-GB, monitoring AChE activity was an ineffective indicator of exposure at miosis levels in these experiments.

54.4 Relationship between regenerated sarin and AChE activity and its use as a dose surrogate

Regenerated sarin S_R from RBCs is experimentally related to AChE activity A, as shown in Fig. 54.3. It is apparent that the relationship can be adequately described in terms of a Michaelis−Menten equation, as follows:

$$A = A_{max}\left[1 - \frac{S_R}{S_R + K_m^{eff}}\right], \qquad (54.1)$$

where $A_{\max} = A_{\max} = 0.623$, and $K_m^{eff} = 0.006$. Note that K_m^{eff} is not the true K_m for GB binding to AChE, which is the concentration of sarin needed to fill half the AChE receptors, since we do not know that the regenerated sarin is the same as the original exposure concentration. (If there were additional sarin binding sites than AChE, for example, the regenerated sarin would underestimate the exposure concentration, and the true K_m would be larger than K_m^{eff} by a certain factor.) The fact that these data fit so well to an M−M type equation strongly suggests that there is a simple proportionality between K_m^{eff} and the "true" K_m, and that sarin regenerated from RBCs is a suitable surrogate for the original exposure to sarin in blood (Fig. 54.3).

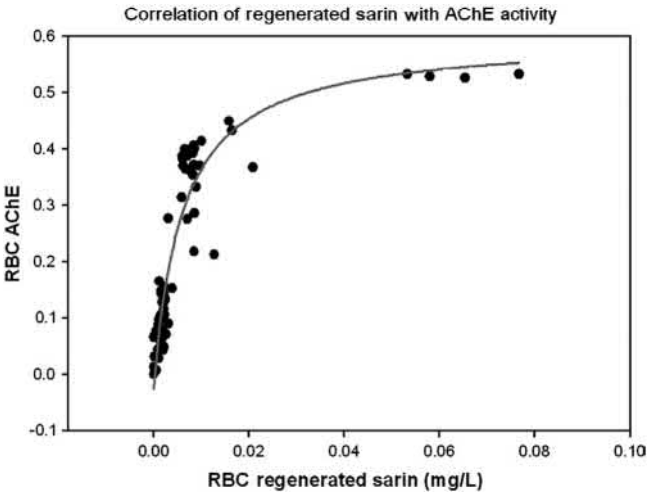

FIGURE 54.3 Plot of RBC AChE activity as a function of regenerated RBC sarin.

Regenerated sarin from RBCs at high doses may exceed the total available binding capacity of the AChE receptors associated with the RBCs. There are, therefore, additional sarin binding sites proposed to be associated with RBCs that continue to bind sarin well after the AChE receptors are filled, and therefore may comprise the bulk of the regenerated sarin at high concentrations. We thus have proposed two competing binding processes: one high-affinity, low-capacity site that dominates the binding of sarin at low concentrations, and which normally releases sarin very slowly, if at all (the AChE receptors); and one relatively low-affinity, high-capacity site, that releases sarin rapidly compared with the circulation time of the blood (Fig. 54.4). This latter site would dominate at higher concentrations, and in the absence of any evidence for its saturation even at these high concentrations, we approximate it as a first-order process described with simple association and dissociation rate constants k_{on} and k_{off}.

54.5 General PBPK model structure

The basic structure of the PBPK model used to describe GB PK and PD followed that of the PBPK-PD model for DFP (Gearhart et al., 1990). Tissue compartments (Fig. 54.2) were added to the previous model structure describing the eye and the skin, where before, these compartments had been lumped together in rapidly perfused or slowly perfused tissues. The eye was added to provide the means of predicting miosis during CW agent exposure, from the systemic absorption of chemicals, but more importantly, from the amount of chemical absorbed directly to the eye structures via the ocular surface via

absorption and diffusion. Adding this compartment also provided a mechanism of addressing the PD effects of sarin on the eye, by correlating inhibition of AChE enzyme levels in the iris with levels of miosis. The skin was added primarily to provide an exposure route for those agents that have a significant dermal absorption potential. Decreases in absorption due to upper respiratory tract (URT) deposition were accommodated by refining the model as shown in Fig. 54.5.

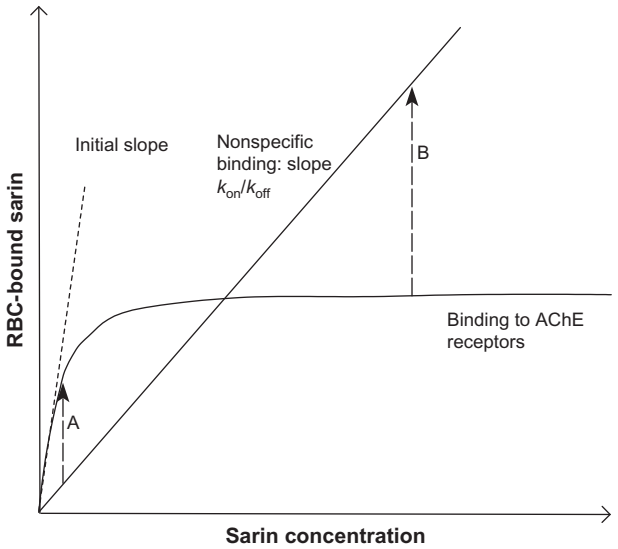

FIGURE 54.4 Hypothetical bound GB versus free sarin concentrations for saturable AChE binding, and linear nonspecific binding. The AChE binding has a higher initial slope, so it will dominate in a competitive situation at low doses (A), but it is saturable, so it will eventually be swamped by the nonspecific binding at higher doses (B).

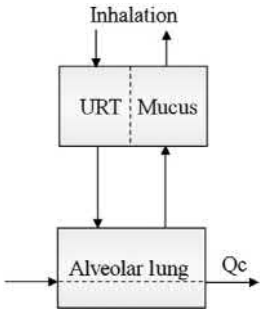

FIGURE 54.5 Detailed schematic for URT interactions for inhalation exposures.

PBPK parameters for individual organ weights were obtained from Breazile et al. (1976); Altman and Katz (1979), and Peeters et al. (1980). Blood flows for most organs were obtained from Peeters et al. (1980) or were scaled from other rodent species. The partition coefficients were based on the values used for DFP (Gearhart et al., 1990) and soman (Langenberg et al., 1997), as well as the cholinesterase and sarinase values for tissue and blood.

The description of the interaction of GB with the mammalian system of the guinea pig followed that as described by Gearhart et al. (1990) for the CWA simulant DFP in rodents and Gearhart et al. (2005) in the Göttingen miniature pig. The description of sarin binding to AChE is shown in Fig. 54.6. Sarin interaction with AChE is described by a bimolecular rate constant, causing loss of AChE activity. The inhibited AChE sites represent the amount of sarin, which can be regenerated from the RBCs.

54.6 PBPK simulation of cholinesterase inhibition and regenerated GB

The main focus of this effort was to develop and validate methods to relate and integrate CWNA toxicity data across routes of exposure in a common species. The specific goal is to compare uptake and clearance kinetics of similar sublethal doses of GB in the blood of guinea pigs exposed to the agent by acute intravenous (IV), inhalation (IH), or subcutaneous (SC) injection. GB regenerated from blood was used as the dose metric to compare the uptake and clearance kinetics of similar doses of GB administered to guinea pigs by an IV, SC, or IH route of exposure. The resulting database will be used to derive a quantitative ratio (SC/IH) of systemically absorbed agent that will allow predictions of the atmospheric GB concentration relevant to a second species based upon subcutaneous injection exposures.

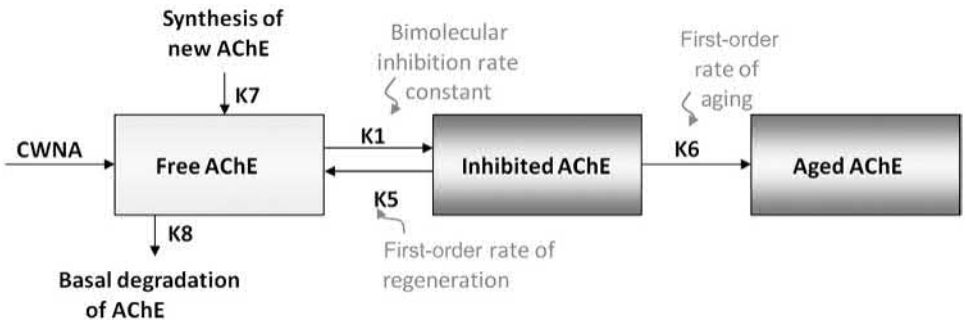

FIGURE 54.6 Schematic for AChE inhibition by sarin.

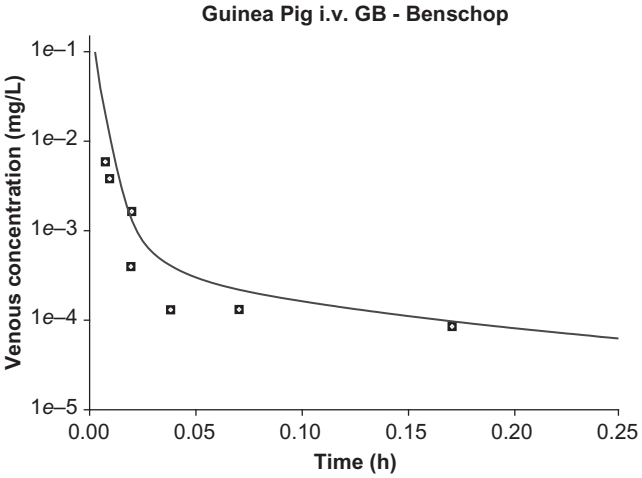

FIGURE 54.7 PBPK-PD simulation of AChE inhibition in the guinea pig after an IV GB dose of $0.8 \times LD_{50}$ (0.0042 mg/kg body weight) (data from Benschop and De Jong, 2001—19.2 μg/kg).

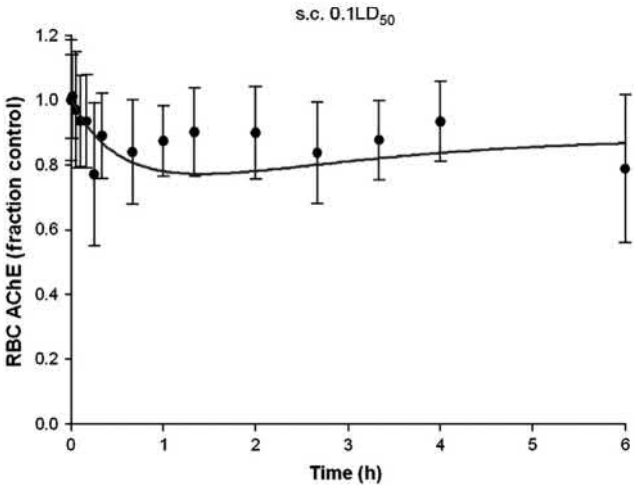

FIGURE 54.8 PBPK-PD simulation of AChE inhibition in the guinea pig after a SC dose of $0.1 \times LD_{50}$ (0.0042 mg/kg body weight).

Guinea pigs were exposed to sublethal levels of GB by IV, IH, and SC routes. Serial blood samples were collected in order to determine simple uptake and clearance kinetics using regenerated agent as a dose metric in a single dose (delivered via IV, SC, and IH). The resulting database was used to derive the PBPK model. This model will be used to determine a quantitative ratio (SC/IH) of a systemically absorbed agent that will allow predictions of the atmospheric GB concentration relevant to the increased guinea pig data sets based upon subcutaneous injection exposures.

Simulation of inhibited cholinesterase and regenerated GB versus the plotted data are exhibited in Figs. 54.7–54.13. The methods of GB exposures or dosing shown in the figures are for either the IV, SC, or IH route. In most cases, the experimental data was well represented by the PBPK-PD model simulations. Those instances where the greatest deviation occurred between the model and the data occurred most likely because of data variability, such that the trend of the data would not follow a reproducible model behavior (Fig. 54.11), or cases where there is still some question about the strength of the model parameters being used. Figs. 54.7–54.10 show the model simulation of some of the key data used to determine model behavior. In each of these cases, the simulations provide a good representation of the overall magnitude and tread of the data. In some cases, as in Figs. 54.7 and 54.9, there are points during the simulation when there is either an overestimation or underestimation of the data. This type of variation between PBPK-PD simulations and data is a common occurrence whenever there is an attempt to exactly simulate time-course kinetic data for such toxicologically active a compound as GB. If it appears that significant decreases or increases in the

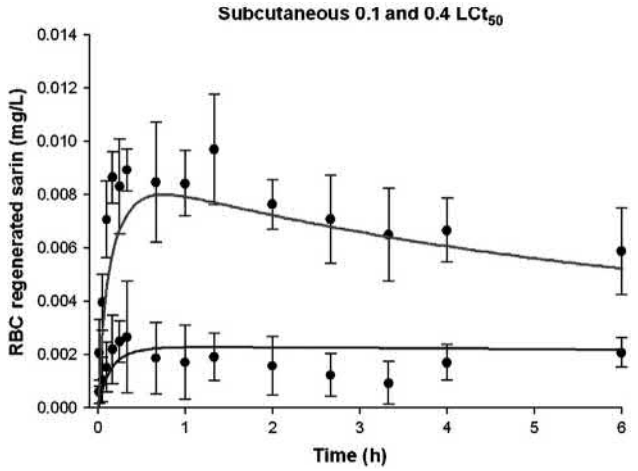

FIGURE 54.9 PBPK-PD simulation of regenerated sarin in the guinea pig after a SC dose of $0.1 \times LD_{50}$ (0.0042 mg/kg body weight) and $0.4 \times LD_{50}$ (0.0168 mg/kg body weight).

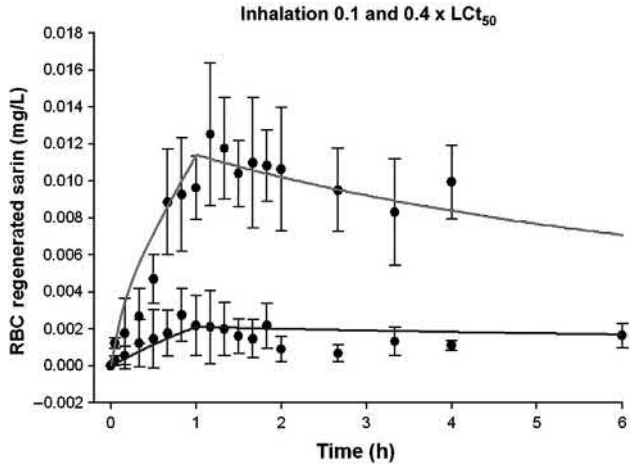

FIGURE 54.10 PBPK-PD simulation of regenerated sarin in the guinea pig after a 1-h IH exposure to sarin vapor at $0.1 \times LC_{50}$ (0.4 mg/m^3) or $0.4 \times LC_{50}$ (1.6 mg/m^3).

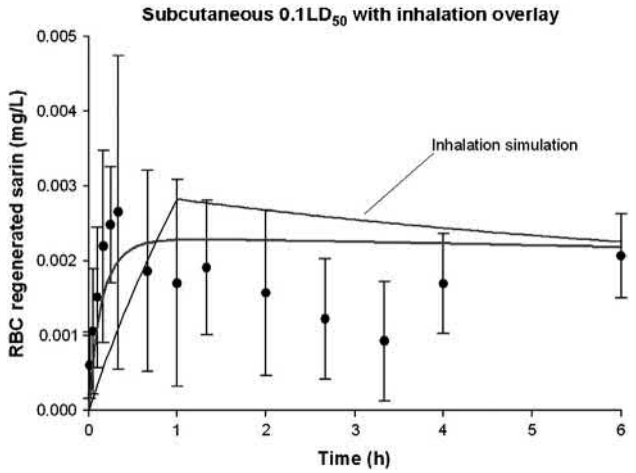

FIGURE 54.11 Overlay of the simulation of regenerated RBC sarin after a $0.1 \times LD_{50}$ SC dose of sarin versus IH dosing simulation at the inhalation concentration required to reproduce the SC data.

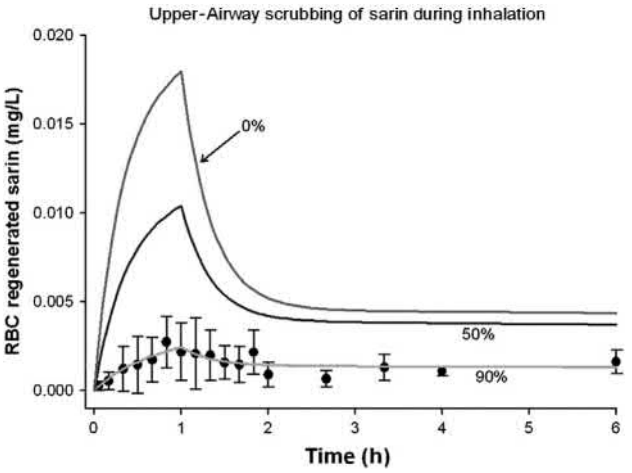

FIGURE 54.13 Upper-airway scrubbing simulations. As was previously seen with the simulation of minipig inhalation exposures, a significant percentage of the inhaled dose was scrubbed by the URT, with the best fit to the data requiring the loss of 90% of the inhaled dose.

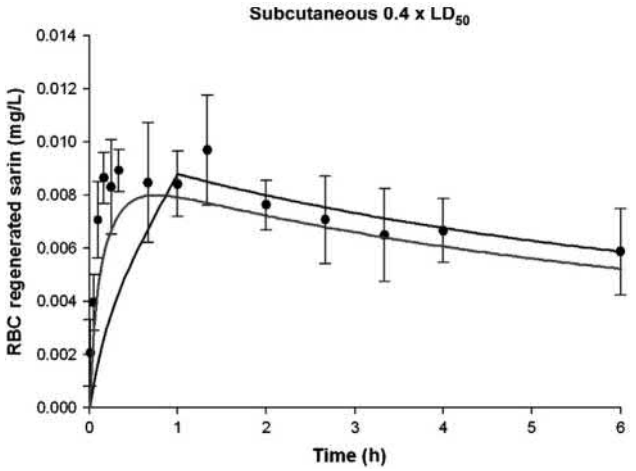

FIGURE 54.12 PBPK-PD simulation of an exposure of guinea pigs to the SC concentration equaling 0.4 of the lethal concentration affecting 50% of the exposure population (LD_{50}).

actual experimental values are indications of real mechanisms and not just animal-to-animal variability, then that may be simulated using more sophisticated methods, as with stochastic processes where actual fluctuations in blood flows or shunting of blood could cause changes in represented concentrations outside the normal trend of the data. Certainly one major issue not addressed in this chapter is the effects of GB on its own PK, which in turn has a significant impact on key PBPK parameters such as blood flow and respiratory ventilation parameters. A well-known response to severe CWNA exposure is a suppression of the cardiac output and respiratory ventilation, both of which could alter measured PK and PD endpoints, and if not modulated in the PBPK-PD model, lead to overestimation or underestimation of data.

54.7 Concluding remarks and future directions

The overall performance of the PBPK-PD model in predicting both the concentration of regenerated GB and the inhibition of RBC AChE was very successful for both the SC and IH routes of exposure at these doses. Previously published studies showing the significant deposition of GB in the URT were confirmed in this analysis of the inhalation of GB in the guinea pig at 0.1 and 0.4 times the LC_{50} for this species. The PBPK-PD model was utilized to equate the delivered dose of GB by SC exposure to what would be obtained after IH exposure. In this analysis, it was determined that at lower GB inhalation concentrations, there is a significant amount of deposition of the agent on the URT (Fig. 54.13), material that is both hydrolyzed and resultantly inactivated, or else it is bound by a mechanism such that it presently is hypothesized to have limited activity. This is one aspect of this analysis that requires further study. To determine the actual IH concentration that would correlate with a particular SC dose, the PBPK-PD model was exercised repeatedly, and the simulation output was overlaid on the SC simulations. This process is shown in Figs. 54.11 and 54.12 for the 0.1 and 0.4 SC LD_{50} levels, respectively. This calculation showed that to reproduce the SC $0.1 \times LD_{50}$ of 0.0042 mg/kg (Fig. 54.11), required an inhalation concentration of 0.08 ppm for a 1-hour inhalation exposure. This value is very close to the experimentally determined value for the inhalation $0.1 \times LC_{50}$ of 0.05 ppm. The SC $0.4 \times LD_{50}$ of 0.0168 mg/kg required an inhalation concentration of 0.28 ppm for a 1-hour inhalation exposure (Fig. 54.12). Further simulations will need to be carried out to confirm these apparent relationships.

A previously developed PBPK-PD model for the CWNA surrogate DFP was parameterized to simulate the concentration and effects of low-level CWAs in the guinea pig after exposure by IH and SC injection. The model code was written to account for absorption of CWAs from multiple sites (respiratory tract—lower and upper, dermal, and ocular) after vapor or SC exposure. References to guinea pig physiology in the literature were used to determine the majority of organ volumes and blood flows, while some parameter values were scaled from other species. Unique features of this PBPK/PD model structure were physiological compartments for the eyes, as a source of external CWA absorption and as a site of cholinesterase binding, and the skin, as a dermal absorption pathway. One initial PD endpoint developed in this model was CWA inhibition of cholinesterases (such as AChE and BuChE). Covalent binding of sarin to cholinesterases and other proteins was also estimated by a novel parameter based on fluoride ion regeneration of the agent. The PBPK/PD model was used to simulate AChE inhibition after inhalation and subcutaneous injection of CWA and to predict potential PD effects at different tissue target sites. This preliminary model will provide a quantitative tool to predict the physiological consequences of low-level, nonlethal exposure to CWNA exposure.

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Chapter 55

Biotransformation of warfare nerve agents

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55.1 Introduction

The first organophosphorus (OP) warfare nerve agents (WNA), tabun (GA) and sarin (GB), were developed in the 1930s by Gerhard Schrader. These, and the even more toxic soman (GD), developed in 1944, are members of the so-called G-agents. Together with VX, developed in 1952 in the United Kingdom, these compounds have emerged as the major WNA known to have been produced and weaponized. The WNA are alkylphosphonic acid esters. Tabun contains a cyanide group. Sarin, cyclosarin, and soman contain a fluorine acyl radical and are methylphosphonofluoridate esters. These WNA contain a C−P bond not found in organophosphate pesticides. The C−P bond is very resistant to hydrolysis. VX contains a sulfur and is an alkylphosphonothiolate.

The mechanism of extremely high toxicity of these agents is excessive cholinergic stimulation caused by inhibition of acetylcholinesterase (AChE) at nervous system synapses. WNA are direct AChE inhibitors reacting rapidly with a serine hydroxyl group in the active site of AChE with the formation of a phosphate or phosphonate ester. The phosphorylated enzyme regenerates very slowly, rendering the enzyme inaccessible for its physiological substrate acetylcholine (ACh). The chirality around the phosphorus atom largely influences the toxicity of these agents as documented in the case of soman whose P(−) isomers are much more toxic than the P(+) isomers (van der Schans et al., 2007; Benschop and De Jong, 1991; Jokanović, 2009a).

Signs and symptoms of the cholinergic syndrome occurring in acute poisoning with WNA and other OP compounds are predictable from their biochemical mechanism of action and are directly related to the levels of AChE activity. After exposure to WNA the symptoms of poisoning occur quickly depending on the dose taken mainly by inhalation (G-agents). These symptoms include miosis (unreactive to light); sweating, rhinorrhea, lacrimation, and salivation; abdominal cramps and other gastrointestinal symptoms; respiratory difficulties and cough; dyspnea, constriction sensation in the chest, wheezing; twitching of facial muscles and tongue, tremors, and fasciculations; bradycardia and ECG changes, pallor, and cyanosis; anorexia, nausea, vomiting, diarrhea, and involuntary urination and defecation. These signs and symptoms are accompanied by central effects such as dizziness, tremulousness, and confusion; ataxia; headache, fatigability, and paresthesia. Finally, seizures, convulsions, twitching, coma, and respiratory failure may occur. Death usually occurs due to respiratory failure resulting from a combination of central and peripheral effects, paralysis of the respiratory muscles, and depression of the brain respiratory center (Karchmar, 2007; World Health Organization, 1986; Marrs and Vale, 2006; Jokanović, 2009a, 2015a, 2018; Jokanović et al., 2011).

In the case of G-agents the intact agent is present in the organism for only a few hours. The dominant metabolic pathway of G-agents is hydrolysis, a process mainly mediated by so-called A-esterases, and the metabolic products formed are corresponding *O*-alkyl methylphosphonic acids in the case of sarin, cyclosarin, and soman (Fig. 55.1). VX is a less suitable substrate for A-esterases. There are also reports discussing a possible role of prolidase (EC 3.4.13.9), a naturally occurring enzyme, in hydrolysis of G-agents. In addition to hydrolysis, binding reactions of WNA to proteins such as AChE, serum cholinesterase (ChE), carboxylesterase (CarbE), albumin, and other macromolecules also occur. Touvrey and coauthors (2019) recently reported the interaction of human bile salt-activated lipase, also denoted as pancreatic lipase, with sarin, tabun, VX, their surrogates, and paraoxon. The interaction of OP with the lipase could represent an additional detoxification mechanism (Jokanović, 2019).

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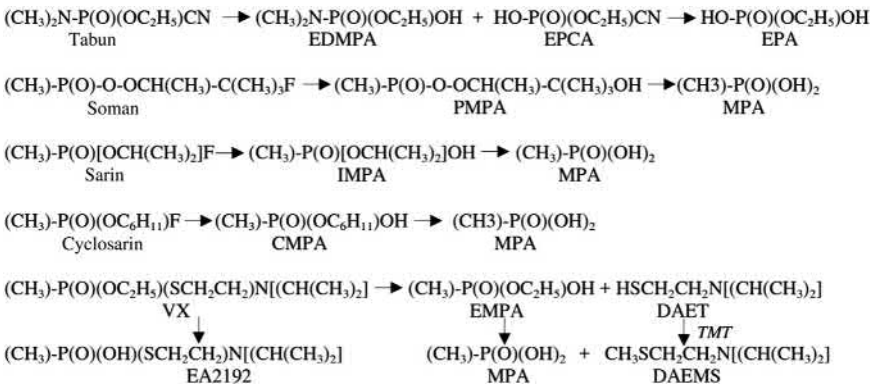

FIGURE 55.1 Metabolic detoxification of tabun, soman, sarin, cyclosarin, and VX. EDMPA, ethyl dimethylaminophosphoric acid; EPA, ethyl phosphoric acid; EPCA, *O*-ethyl-cyanophosphoric acid; IMPA, isopropyl methylphosphonic acid; PMPA, pinacolyl methylphosphonic acid; CHMPA, cyclohexyl methylphosphonic acid; EMPA, ethyl methylphosphonic acid; MPA, methylphosphonic acid; DAET, 2-(diisopropylamino) ethanethiol; DAEMS, 2-(diisopropylaminoethyl) methylsulfide; *TMT*, thiol S-methyltransferase (EC 2.1.1.9) (Nakajima et al., 1998; Katagi et al., 1999; Jokanović, 2001, 2009b, 2015b; Evans et al., 2008; Black, 2010; Tenberken et al., 2010; Reiter et al., 2011; Black and Read, 2013; Kranawetvogl et al., 2013; Son et al., 2019).

The toxicological importance of binding reactions of WNA to these proteins is in the decrease in free unbound concentration of WNA in blood, which causes less AChE inhibition in the nervous system. Both WNA and OP pesticides lose their acyl radicals when they react with AChE, ChE, and CarbE and other proteins. After binding to AChE and ChE the phosphoryl residues of soman, sarin, cyclosarin, tabun, and VX undergo an intramolecular rearrangement with subsequent loss of one phosphoryl group known as the aging reaction.

In addition, due to the reversibility of the binding reaction of sarin and soman to CarbE, it appears that CarbE is involved in the metabolic detoxification of these agents to their corresponding nontoxic metabolites, isopropyl methylphosphonic acid (IMPA) and pinacolyl methylphosphonic acid (PMPA) (Jokanović et al., 1996; Jokanović, 2009b, 2015b).

One of the important proofs which support the significance of detoxification reactions of WNA in the body was presented by Fonnum and Sterri (1981), who reported that only 5% of LD_{50} of soman in rats or about 5 μg/kg reacts with AChE causing acute toxic effects, while the remaining 95% passes through various metabolic reactions.

In this chapter the mechanisms involved in biotransformation of WNA are discussed. Mechanisms of biotransformation of OP pesticides are beyond the scope of this chapter and interested readers are referred to other publications (Jokanović, 2001, 2009a; Chambers et al., 2001; Tang et al., 2006).

55.2 Chemical aspects of biotransformation of nerve agents

The G agents are anticholinesterase substances that at sufficient concentrations can be toxic or fatal by any route of exposure. Differences in volatility and water solubility result in varied degrees of persistence and variations in the likelihood of exposure by certain routes. Of the G agents, tabun gives rise to the greatest number of degradation products. The metabolites of tabun, EDMPA and EPCA (Fig. 55.1), are apparently unstable, while the EPA is an excretion metabolite of tabun as well as of certain pesticides and plasticizers (Tenberken et al., 2010; Black and Read, 2013; Jokanović, 2015b). The final metabolite of tabun is a phosphate (Black, 2010). Toxicity data are available only for a limited subset of the tabun degradation products. One of the tabun hydrolysis products, dimethylamine, is moderately toxic in terms of acute lethality but causes irritation of the human respiratory tract (Munro et al., 1999).

Sarin is metabolized to IMPA, which slowly undergoes further hydrolysis to the very stable MPA. IMPA also forms in the course of spontaneous reactivation of sarin-inhibited CarbE and ChEs. IMPA has shown low toxicity in rats and mice in all toxicity tests conducted except mild skin irritation in rabbits. Limited data suggest that MPA is nontoxic in terms of acute oral toxicity in the rat and mouse, but it showed irritant effects in the human eye and skin (Munro et al., 1999; Jokanović, 2009a, 2015b; Son et al., 2019; Young and Capacio, 2019).

In the study of Little et al. (1986) a single sublethal dose (80 μg/kg) of radiolabeled sarin was administered intravenously to mice and the tissue distribution was followed for 24 h. Within 1 min, sarin was distributed to the kidneys, lungs, brain, lungs, heart, and diaphragm. Thereafter, the concentrations in all tissues rapidly declined and within 15 min only trace quantities of [^{3}H] sarin were found in the brain. Within the first minute, about half of the labeled sarin was in the form of IMPA. The highest concentrations of sarin and its metabolites

were found in the kidneys. Lower concentrations of IMPA and MPA were detected in the liver, suggesting a minor role for the liver in the detoxification of sarin.

Shih et al. (1994) injected rats subcutaneously with a single dose of 75 μg/kg of sarin, cyclosarin, and soman and measured excretion of the hydrolyzed metabolites, the alkylmethylphosphonic acids, including IMPA and corresponding MPAs. MPA was a major and common metabolite of the three compounds. Urinary excretion over the first 24 h accounted for approximately 90% of the administered doses of sarin and cyclosarin. Almost total recoveries of the given doses for sarin and cyclosarin in metabolite form were obtained from the urine. Urinary elimination was found to be rapid and the terminal elimination half-life of sarin metabolites in urine was 3.7 h. Most of the administered dose of sarin was retrieved from the urine in metabolite form after 2 days. The terminal elimination half-life of cyclosarin in urine was 9.9 h. Soman metabolite showed a biphasic elimination curve with terminal half-lives of 18.5 and 3.6 h. Soman was excreted at a slower rate with a recovery of only 62%. The first phase of elimination of soman results from enzymatic hydrolysis of the inactive P(+) isomers, and the slower phase is from the active P(−) isomers (Benschop and De Jong, 1991). The elimination study in rats determined IMPA in blood up to 14 h post-exposure, CHMPA up to 2 days, and PMPA up to at least 3 days.

The distribution, metabolism, and elimination of sarin in humans appear to resemble the findings in animals. Minami et al. (1997, 1998) detected the sarin metabolite IMPA in urine of humans after the terrorist attack in Tokyo in 1995. They found peak levels of IMPA or MPA in urine 10−18 h after exposure. The levels of IMPA in urine correlated with the clinical symptoms. They also found evidence of distribution of sarin to the human brain in four of the 12 people who died after exposure. IMPA and MPA were detected in the urine of a patient poisoned with sarin in Matsumoto (Nakajima et al., 1998).

Biotransformation products of soman include pinacolyl methylphosphonic acid (PMPA) and MPA. No biologic data were found for PMPA (Munro et al., 1999; Jokanović, 2009b). It has been shown that the toxic C(±)P(−)-isomers of soman react rapidly with covalent binding sites. The less toxic C(±)P(+)-isomers are hydrolyzed several orders of magnitude faster than the C(±)P(−)-isomers. The low toxicity of the C(±)P(+)-isomers is primarily due to a low intrinsic reactivity toward AChE and rapid metabolic hydrolysis (van der Schans et al., 2007). The levels of C(±)P(−)-isomers remain toxicologically relevant for periods of 50−100 min in rats, guinea pigs, and marmosets at doses of 2−3 LD_{50} (Benschop and De Jong, 1991).

VX is a potent anticholinesterase agent that can act by dermal, oral, and inhalation routes of exposure. There are certain characteristics of VX which make the agent different from G-agents: (1) VX is present in blood as protonated amine; (2) it is hydolyzed at a much slower rate than G-agents; (3) it reacts more slowly with CarbE (due to its positively charged quaternary ammonium group) and A-esterases, and (4) VX can be metabolized by other routes such as oxidation reactions at nitrogen and/or sulfur (Black, 2010; Jokanović, 2009b, 2015b). A few of the metabolic products may retain some anticholinesterase activity (such as EA2192) (Munro et al., 1999), but hydrolysis of one or more alkyl ester bonds of organophosphonic acids results in generally nontoxic alkyl methylphosphonic acids EMPA and MPA. MPA is resistant to further hydrolysis. Munro et al. (1999) discussed the metabolic degradation products of VX in mammals and found that there are about 25 such products and each had shown different levels of toxicity.

There are three phosphorus-containing metabolites of VX: EA2192 (which has shown some anticholinesterase activity), EMPA, and MPA (not AChE inhibitors) (Fig. 55.1). EMPA appears to be the major metabolite of VX in urine (Black and Read, 2013). The presence of EA2192 metabolite in humans is important since it retains the 2-diisopropylaminoethylthio substituent that confirms exposure to VX (Black and Read, 2013). After intravenous and dermal administration of sublethal doses of VX to swine both VX and EA2192 could be quantified during 540 min after exposure (Reiter et al., 2011). In addition, there are two nonphosphorus metabolites of VX—DAET and its methylation product DAEMS identified in human serum from the victim of the Osaka VX accident (Tsuchihashi et al., 1998). The conversion from DAET to DAEMS is catalyzed by thiol S-methyltransferase (EC 2.1.1.9) (Reiter et al., 2011).

Benschop et al. (2000) and van der Schans et al. (2003) studied the toxicokinetics of VX stereoisomers in hairless guinea pigs and marmosets. Following an intravenous dose of 28 μg/kg (marmosets) or 56 μg/kg (guinea pigs), VX was found in the blood at toxicologically relevant levels even after 6 h. Detoxification proceeded at a slower rate in marmosets than in guinea pigs. The VX metabolite EMPA (Fig. 55.1) was found in the blood of the exposed animals; however, the metabolite contributed only 5% to the recovery of the phosphonyl moieties related to the VX dose. Metabolites of VX were also evaluated in in vitro studies by treating liver homogenates and plasma from hairless guinea pigs, marmosets, and humans with the radio-labeled compounds, ^{35}S-VX. The potential toxic metabolite VX-*N*-oxide was not found. Desethyl-VX was found after incubation of VX in plasma of all three species; however, because of its slow rate of formation, Benschop et al. (2000) concluded that it would be unlikely that VX would be present at toxicologically relevant levels after administration of VX in vivo. In vitro

studies with ^{35}S-VX revealed that a significant part of the thiol-containing leaving group (*S*-2-[*N*,*N*-diisopropylamino]ethane thiol) was bound to albumin.

Tsuchihashi et al. (1998) developed a method for identification of VX metabolites in serum collected from a victim of the Osaka VX incident. In the serum sample, both EMPA and 2-(diisopropylamino-ethyl)methylsulfide (DAEMS) were detected. The techniques using GC-MS and GC-MS-MS were applicable to biological samples such as serum. These results provided the first documented identification of the specific metabolites of VX in victim's serum and clarified a part of the metabolic pathway of VX in the human body. In addition, methods have been developed for measuring the VX-inhibited AChE hydrolytic product EMPA (Noort et al., 1998, 2002).

55.3 Esterases involved in the metabolism of warfare nerve agents

Numerous esterases can react with OPs but in a different way. The first classification was given by the late Professor Aldridge (1953). In the first group there were esterases that hydrolyze OPs as substrates, and among them particularly their uncharged esters, that are not inhibited by these compounds. This group of enzymes was called *A-esterases*, although in the literature there are many other names for the same group of enzymes given according to the substrate hydrolyzed (paraoxonase, somanase, sarinase, etc.) or their chemical structure (phosphotriesterases, phosphorylphosphatases, anhydrases of OP compounds). In the second group of enzymes interacting with OPs were *B-esterases*, which are inhibited with OPs in the progressive reaction that is time- and temperature-dependent. This group of enzymes comprises AChE, ChE, CarbE, trypsin, chymotrypsin, and other enzymes. In the third group were *C-esterases* that do not interact with OP. It is paradoxical, but basically true, that OPs can be substrates for both A- and B-esterases because their concentration in blood and tissues is decreased in the presence of these enzymes (Jokanović, 2009b).

The mechanisms of interaction of A- and B-esterases with WNA and other OPs are similar. B-esterases initially form Michaelis complex with an OP inhibitor producing phosphorylated or inhibited enzyme that either reactivates very slowly or does not reactivate at all. However, after formation of Michaelis complex with OP A-esterases catalyze hydrolysis of OP and their catalytic activity and turnover rate are very high.

55.3.1 A-esterases

In a classification from 1992 (International Union of Biochemistry, 1992) hydrolases of OP were described as a special entity of "phosphoric triester hydrolases," which comprises three groups of enzymes: (1) phosphoric monoester hydrolases (EC 3.1.3), (2) phosphoric diester hydrolases (EC 3.1.4), and (3) phosphoric triester hydrolases (phosphotriesterases) (EC 3.1.8). Phosphoric triester hydrolases are further divided into two similar subgroups: aryldialkylphosphatases (EC 3.1.8.1) and diisopropylfluorophosphatases (EC 3.1.8.2).

Aryldialkylphosphatases take part in hydrolysis of aryldialkylphosphates producing dialkylphosphate and aryl alcohol. These enzymes react with substrates such as paraoxon (Fig. 55.2), but also with phosphonates and phosphinates. Other names for this group of enzymes are hydrolases of OPC, A-esterases, paraoxonases, PON1, aryltriphosphatase, and aryltriphosphate dialkylphosphohydrolase. They are inhibited with compounds that form chelates like EDTA since they require the presence of divalent ions, mainly Ca^{2+} (Walker, 1993). Some fractions of the enzyme purified from human serum were able to hydrolyze both paraoxon and phenylacetate and it was thought for a long time that the same enzyme was responsible for both. However, enzymes hydrolyzing aryl esters are classified as arylesterases (EC 3.1.1.2).

Diisopropylfluorophosphatases take part in hydrolysis of diisopropylfluorophosphate (DFP) and similar compounds producing diisopropylfluorophosphoric acid and fluoride ion (Fig. 55.2). These enzymes react with phosphorus anhydride bonds such as those between phosphorus and acyl radical (F^-, Cl^-, CN^-) in highly toxic OPC such as soman, sarin, tabun, and DFP, and they were accordingly named somanase, sarinase, tabunase, DFPase, diisopropylfluorophosphate fluorohydrolase, OPA anhydrase, OPAA, and OP acid anhydrolase. They are also inhibited by chelating agents, and their activity requires the presence of divalent ions such as Mg^{2+}, Mn^{2+}, Co^{2+}, and Cd^{2+}, but not Ca^{2+} (Walker, 1993; Kondo et al., 2004). They exist in several forms, even in different tissues of the same species, which react differently with substrates.

In the literature there are papers reporting an important role of senescence marker protein-30 (SM-30) in the

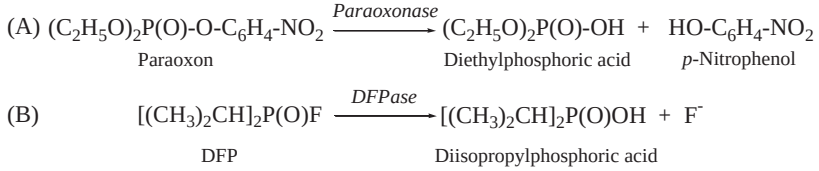

FIGURE 55.2 (A) Hydrolysis of paraoxon with aryldialkylphosphatase (paraoxonase, EC 3.1.8.1) and (B) hydrolysis of diisopropylfluorophosphate (DFP) with diisopropylfluorophosphatase (DFPase) (EC 3.1.8.2).

hydrolysis of diisopropylfluorophosphate (DFP) and similar substrates (Kondo et al., 2004; diTargiani et al., 2010). SM-30 protein was given the code EC 3.1.8.2, which was previously assigned to DFPase. After checking all available data it appears that both SM-30 and DFPase are the same enzymes and that their names are synonyms! DFPase is, therefore, a 34-kDa metalloprotein expressed predominantly in the liver and kidney of mammals. Although the identity of the metal ion is not clearly established, it was shown that the activity of purified DFPase from rat liver was stimulated by $MgCl_2$, $MnCl_2$, $CoCl_2$, and $CdCl_2$ (Kondo et al., 2004). It has been linked with the regulation of calcium homeostasis and the protection of cells from apoptosis. Consistent with these observations, it was found that the sequence of DFPase was identical to that of a Ca^{2+}-binding protein, regucalcin. Rat liver DFPase was shown to hydrolyze DFP, phenyl acetate, and gluconolactones. In vivo studies with SMP-30 knockout mice confirmed its role in the hydrolysis of DFP in the liver of these animals (diTargiani et al., 2010). A reduction in DFPase expression might account for the age-associated deterioration of cellular functions and enhanced susceptibility to harmful stimuli in aged tissue (Kondo et al., 2004).

In the literature published during the last 40 years the term PON1 apparently covers both phosphoric triester hydrolases. In further text, in order to avoid possible confusion, the term A-esterase will be generally used for enzymes hydrolyzing OPs along with other terms (such as PON1, paraoxonase, etc.) as they appear in original references.

The molecular weight of human A-esterases is between 43 and 45 kDa. Human A-esterase is a protein of 355 amino acids having two polymorph sites in which arginine or glutamine are located at position 192 and methionine or leucine at position 55 (La Du et al., 1993; Li et al., 1995). The former accounts for three genotypes (QQ, RR, and QR) relating to the catalytic properties of two forms of an enzyme (types R and Q allozymes), which hydrolyze certain organophosphates at different rates. Its three-dimensional structure is also known (Benning et al., 1994; Vilanova and Sogorb, 1999). At the active site of A-esterases there are two metal cations connected via a common ligand, and most of the other protein groups are bound to this binuclear site through imidazolium side chains from histidine groups (Benning et al., 1994). The activity of these enzymes largely depends on Ca^{2+} that represents a necessary factor for maintaining the function of the active site and it is also possible that Ca^{2+} directly participates in catalytic reactions or maintain the conformation of amino acids at the active site. In addition, in the case of paraoxon, Ca^{2+} facilitates the release of diethylphosphate from the active site, probably by polarizing bond P═O, which makes the phosphorus atom much more sensitive to the nucleophilic attack of hydroxyl ions (Vitarius and Sultatos, 1995; diTargiani et al., 2010). Human A-esterases can hydrolyze many OPs, among them paraoxon, soman, sarin, tabun, chlorpyriphos and chlorpyriphos oxon, DFP, dichlorvos, diazoxon, and pyrimiphos methyl oxon.

The R allozyme (Arg192) hydrolyzes the organophosphate paraoxon at a high rate; however, it has a low activity toward sarin, soman, and diazoxon. Lower activity means that more sarin would be bioavailable to exert its anticholinesterase effects. The Q allozyme, on the other hand, has high activity toward sarin, soman, and diazoxon (and low activity toward paraoxon). Thus, individuals with the Q allozyme (QQ or QR) are expected to have greater hydrolysis of sarin than individuals homozygous for the R allele (RR) (Costa et al., 1999, 2005). In Caucasian populations, the frequency of the R allele is about 0.3, but the frequency is 0.66 in the Japanese population (Yamasaki et al., 1997; Costa et al., 2006). This would make individuals in the Japanese population more sensitive to the toxicity of sarin, a fact that may have contributed to their morbidity and mortality after the terrorist attacks in 1995. The concentration of PON1 in human plasma (about 60 mg/L) varies between individuals by as much as 13-fold. The PON1 activity level is determined by a combination of complex genetic interactions and environmental/dietary factors, giving rise to a 40-fold variation in PON1 for a single individual (Rochu et al., 2007). The relationship between paraoxonase polymorphisms and toxicity of OP compounds was further discussed in an excellent article by Costa et al. (2006) and other papers from his team.

Paraoxonase (PON1) is a member of a family of proteins that also includes PON2 and PON3, the genes of which are placed on the long arm of human chromosome 7 (q21.22). PON2 and PON3 have about 65% homology to PON1, but they cannot hydrolyze WNA and other organophosphates (Draganov et al., 2005; Aviram and Rosenblat, 2008). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoprotein (HDL) particles (Costa et al., 2005). The crystal structure for a recombinant PON1 indicates that it is a six-bladed β-propeller, with two calcium ions in the central tunnel, one of which is essential for enzyme activity and the other for enzyme stability (diTargiani et al., 2010). The primary physiological role of PON1 appears to be protection of low-density lipoproteins (LDL) from oxidative modifications (Mackness et al., 1993; Aviram et al., 1998; Vilanova and Sogorb, 1999; Rochu et al., 2007). Human PON1 is apparently involved in drug metabolism and in preventing atherosclerosis (diTargiani et al., 2010). Endogenous PON1 is associated with HDL that represents its main carrier in blood and this association has been considered

as a crucial element in the increased stability and half-life of PON1 in the circulation (Gaidukov et al., 2009; Veliyaveettil et al., 2012). In addition to its role in lipid metabolism and cardiovascular disease and atherosclerosis, PON1 has been shown to play a role in the metabolism of drugs and xenobiotics containing aromatic carboxyl esters (Costa et al., 2003). It is also suggested that PON1 hydrolyzes various lactones, including naturally occurring lactone metabolites.

The differences in the activity of A-esterases due to polymorphisms may have an important effect on the toxicity of OP in humans who are occupationally or accidentally exposed. In this respect, it was proposed that humans expressing lower activity of A-esterases could be more susceptible to the toxic effects of OP and there were suggestions that such individuals should not be exposed (Mackness, 1989). Hernandez et al. (2004) have confirmed this hypothesis, suggesting the association of paraoxonase phenotypes with susceptibility of humans to OP pesticide toxicity. However, additional studies are needed to fully understand the effects of A-esterase polymorphism on the capacity of detoxification and toxicity of OP.

55.3.1.1 Toxicological relevance of A-esterases

A-esterases purified from *Pseudomonas diminuta* given to mice decreased inhibition of AChE in brain and ChE in serum in poisoning with paraoxon and DFP, and this effect was less significant in poisoning with soman and sarin (Tuovinen et al., 1994). Purified A-esterases have shown a protective effect when given to mice before poisoning with tabun (Ashani et al., 1991a).

It was reported that PON1 preferentially hydrolyzes the less toxic soman enantiomers (Yeung et al., 2008). Injection of human PON-1 was found to protect guinea pigs against 1.2 LD_{50} of racemic sarin and soman (Veliyaveettil et al., 2012).

Haley et al. (1999) investigated PON1 genotype and serum enzyme activity in a group of 25 ill Gulf War veterans and 20 controls. Ill veterans were more likely than controls to possess the R allele (QR heterozygotes or R homozygotes) and to exhibit lower enzyme activity. This study raised the possibility that the R genotype (low sarin-hydrolyzing activity) may represent a risk factor for illness in Gulf War veterans. However, because of the very small number of participants included in the study, such findings require confirmation in a larger population (Furlong, 2000a). La Du et al. (2001) have also found reduced sarinase and somanase levels in plasma obtained from Gulf War veterans. In a similar study, in a group of 152 UK Gulf War veterans, the PON1 activity was lower in veterans than in a control group, but the differences were independent of PON1 genotype (Mackness et al., 2000). However, in both studies there were no indications about the extent of exposure to nerve agents and the possible effects of such exposures.

55.3.2 B-esterases

B-esterases are the group of enzymes that can be inhibited by OP compounds in the progressive reaction that is time- and temperature-dependent. This group of enzymes comprises AChE (EC 3.1.1.7), ChE (EC 3.1.1.8), CarbE (EC 3.1.1.1), chymotrypsin, trypsin, and some other enzymes. A common feature of these enzymes is that they have a serine hydroxyl group at the active site that enables them to react with OP in a similar fashion. AChE, ChE, and CarbE are members of the α/β hydrolase family and have a high degree of overall homology in their amino acid sequences, but they differ in several critical regions that produce differences in their biochemical properties. The most significant biochemical differences in these esterases are related to the extent of aging of the OP-inhibited esterase, the size of the active site, and the ability of the OP-inhibited enzyme to undergo spontaneous or oxime-induced reactivation (Doctor et al., 2001).

55.3.2.1 Serum cholinesterase

While the biological role of ChE is still unclear, it is known that soman, sarin, tabun, and VX bind to ChE, without any apparent toxic effects, decreasing the amount of free WNA available for inhibition of AChE in the central nervous system and erythrocytes (Jokanović, 2009a). Pretreatment with human plasma ChE (hBChE) has protected laboratory mice (Ashani et al., 1991b) and monkeys (Raveh et al., 1997) from lethal and other acute toxic effects of VX exposure. Human BChE is now in an advanced stage of development as a bioscavanger and is close to obtaining marketing approval as a pretreatment for OP poisoning (Saxena et al., 2011a; Trovaslet-Leroy et al., 2011). It is estimated that a dose of 200 mg is required to protect a human against 2 LD_{50} of soman (Saxena et al., 2011a). hBChE reacts with OP to form the 1:1 complex and an i.m. or i.v. injection protects animals against 3−5 LD_{50} soman, tabun, and VX (Lenz et al., 2007). A dose up to 250 mg/70 kg of hBChE is able to protect humans from 1 LD_{50} of OP (Ashani and Pistinner, 2004). Such a dose of hBChE is required because of the stoichiometric and irreversible reaction between hBChE and OP (Trovaslet-Leroy et al., 2011). Animal studies have shown that administration of large doses of hBChE provide protection against up to 5.5 LD_{50} of soman or 8 LD_{50} of VX (Saxena et al., 2011a). Pretreatment with 7.5 mg/kg completely prevents toxicity in minipigs exposed to sarin by inhalation (Saxena et al., 2011b). hBChE derived from plasma is present in the blood of guinea pigs up to 4 days without any signs of toxicity

(Saxena et al., 2011a) and without any adverse effect in rats (Nachon et al., 2013). The behavioral and physiological safety of plasma hBChE was established at 21 mg/kg in mice (Ilyushin et al., 2013) and 30 mg/kg in rhesus monkeys (Myers et al., 2012).

The structure, function, genetic variants, potential uses of human butyrylcholinesterase, and its role in toxicology of OPs have been reviewed by Lockridge (2015) and Masson and Nachon (2017).

ChE is a serine esterase glycoprotein, composed of four 85-kDa subunits, synthesized mainly in the liver, which does not have any known physiological function. From the liver, ChE is secreted to plasma and its activity correlates with the physiological and/or pathological condition of the liver. ChE can be found in more than 40 genetic variants differing in their susceptibility to chemicals (Jokanović and Prostran, 2009). ChE may have roles in cholinergic neurotransmission (Mesulam et al., 2002), other nervous system functions (cellular proliferation and neurite growth during the development of the nervous system), and in neurodegenerative disorders (Darvesh et al., 2003).

Variability in plasma ChE activity is a parameter of concern for characterization of population susceptibility to nerve agent exposure. Selective inhibition of AChE i ChE had no effect on acute soman toxicity to mice (Clement, 1984). Since VX reacts with ChE, and very slowly with CarbE (due to its positively charged quaternary ammonium group), it appears that ChE may have a significant role in detoxification of VX. Wide variations in ChE activity seen in individuals not exposed to OPs, caused by genetic, physiological, and pathological conditions, as well as interactions with many drugs, may strongly influence the susceptibility of those individuals to OP compounds (Jokanović and Maksimović, 1997). It is possible that individuals with lower ChE activity may be more susceptible to the effects of OPs, including nerve agents.

55.3.2.2 Carboxylesterases (EC 3.1.1.1)

The mammalian CarbEs comprise a multigene family whose gene products are located in the endoplasmic reticulum (Hosokawa and Satoh, 2006). CarbEs are the enzymes that hydrolyze esters and thioesters or amide groups of carboxylic acids. They are also mentioned in the literature as aliesterases and esterase D. CarbE have a very important role in metabolism of lipids, endogenous fatty acids, esters, steroids, and a large number of ester-containing drugs and prodrugs such as salicylates, clofibrate, procaine, lorazepam, cilazapril, and other angiotensin-converting enzyme inhibitors, narcotics (cocaine, heroin), and capsaicin. CarbEs also participate in detoxification of pesticides (carbofuran, pyrethroids, OPs), acrylates, mycotoxins (T2 toxin), and esters of nicotinic acid (Cashman et al., 1996). Certain isoenzymes of hepatic microsomal CarbE are involved in the metabolic activation of some carcinogens and are associated with hepatocarcinogenesis (Hosokawa and Satoh, 2006). Similar enzymes to CarbE are arylesterases (EC 3.1.1.2) that hydrolyze aromatic esters of carboxylic acids. However, this classification is not perfect since CarbEs hydrolyze some aromatic esters (i.e., phenyl valerate, phenyl butyrate) and arylesterases hydrolyze certain aliphatic esters. These two enzymes can be clearly differentiated according to their interaction with OP since CarbEs are inhibited with OPs while arylesterases can hydrolyze some OPs that contain an aromatic group, such as paraoxon and chlorpyriphos oxon, and because of this they were sometimes confused with A-esterases.

The CarbE profile in humans is not well known. While CarbEs were considered to be absent from the blood plasma of humans (Li et al., 2005), they are, indeed, present in human erythrocytes and monocytes as well as in human liver, kidney, lung, skin, and nasal tissue (Cashman et al., 1996). Additional literature documents the presence of CarbEs in many human tissues and fluids, including brain, milk, mammary gland, pancreas, small intestine, colon, stomach, placenta, and plasma and serum (Chanda et al., 2002; Kaliste-Korhonen et al., 1996). The lung CarbEs are associated with alveolar macrophages (Munger et al., 1991). Further, CarbEs are present in human tissues and organs where exposure to nerve agent vapors would likely first occur (nasal tissues and the lung), would be distributed (erythrocytes, monocytes, plasma), and would generate effects (brain, stomach, colon, etc.). Chanda et al. (2002) indicate that full characterization of the OP-protective capabilities of CarbEs requires assessment not only of the amount but also of the affinity exhibited by CarbEs for the inhibitor as well as the total CarbE activity unlikely to be inhibited. The detoxification potential of CarbEs is apparently complex and is an area requiring further studies.

CarbEs are proteins of molecular weight between 47 and 65 kDa that can be found in the microsomal fraction of many mammalian tissues (Satoh and Hosokawa, 1998). CarbEs are synthesized in the liver and secreted into plasma (via the Golgi apparatus) where they are present in soluble form. Their physicochemical and immunological properties and the sequence of amino acids are very similar, while their specificity toward various substrates is different (Hosokawa et al., 1995; Satoh and Hosokawa, 1998). CarbEs belong to the group of esterases having serine at their active site that hydrolyze esters of carboxylic acids in a biphasic reaction. In the first phase, carboxylic ester acylates the hydroxyl group of serine at the active site, and in the second phase serine is being deacylated in the presence of water (Augustinsson, 1958).

The active site of CarbE comprises a peptide isoleucine-phenylalanine-glycine-histidine-serine-methionine-glycine-glycine, with serine and histidine directly participating in biochemical reactions. The physiological substrate for CarbE is probably *O*-acetyl sialic acid (Satoh and Hosokawa, 1998). CarbE can be differentiated from other serine esterases AChE (EC 3.1.1.7) and ChE (EC 3.1.1.8) in that AChE and ChE react with positively charged esters such as acetylcholine and butyrylcholine and can be inhibited with carbamates, while CarbE does not react with positively charged esters and inhibition with carbamates occurs only at high concentrations. Inhibition of CarbE does not cause any known toxic effects. The classification and nomenclature of CarbEs were proposed by Satoh and Hosokawa (1998) and readers are also referred to an excellent book chapter (Hosokawa and Satoh, 2006) for more details.

55.3.2.3 The relationship between CarbE activity and toxicity of warfare nerve agents

Several studies have shown that triorthocresyl phosphate (TOCP) and its active metabolite CBDP (2-/*o*-cresyl/-4H-1:3:2-benzodioxaphosphorin oxide) (specific irreversible inhibitor of CarbE and weak anticholinesterase agent) potentiate toxicity of soman, sarin, and tabun (Bošković, 1978; Clement, 1984; Jokanović, 1989) but not of VX agent (Bošković, 1978), probably because VX in physiological conditions is positively charged and a weak inhibitor of CarbE (Maxwell, 1992). Bošković (1978) found that pretreatment of mice with CBDP increased the s.c. toxicity of soman 19.1-fold, and its i.p. toxicity 17.8-fold. For other nerve agents he observed an increase of s.c. toxicity of sarin, tabun, and VX of 11-, 5-, and 0.24-fold, respectively. Clement (1984) observed that the potentiation of soman toxicity in mice after previous administration of TOCP or CBDP was directly related to plasma CarbE and not to activity of CarbE in liver and other tissues. This effect of TOCP and CBDP was explained by phosphorylation of active sites at CarbE that occupies the binding sites for other OPs, increasing their concentration in the circulation and therefore their acute toxicity (Jokanović, 2001, 2009b, 2015b). Binding of soman to CarbE in rodents occurs specifically with the most toxic stereoisomer of the agent (Cashman et al., 1996).

The detoxification potential of endogenous CarbE to protect against the lethal effects of WNA exposure was tested by Maxwell (1992), who observed that a wide range in potentiation of toxicity of different OPs in vivo cannot be correlated with reactivity of these compounds toward CarbE, showing that soman toxicity in rats with inhibited CarbE was potentiated sixfold, respectively, in spite of their similar inhibitory power for CarbE. It was concluded that detoxification of OP via CarbE is very important for highly toxic OPs such as soman, sarin, tabun, and paraoxon with LD_{50} of < 2 μmol/kg, while it is less important for less toxic OPs such as DFP ($LD_{50} = 9.75$ μmol/kg) and dichlorvos ($LD_{50} = 98.4$ μmol/kg). Having in mind that relatively higher concentrations of OP insecticides have to be achieved in the circulation and tissues in order to exert toxicity, a dominant factor in detoxification of less toxic OPs is A-esterases as their catalytic activity is proportional to substrate concentration and their K_m value is in the millimolar range (Jokanović, 2009b, 2015b).

Contrary to these findings of decreased CarbE activity increasing toxicity of many OPs, there are also data showing that increased CarbE activity can decrease the toxicity of OPs. CarbE activity can be increased by about 80% after repeated administration of phenobarbital to rats and mice by a mechanism of enzyme induction which caused a decrease in soman and tabun toxicity by twofold, while toxicity of sarin was not affected, probably because plasma CarbE inhibited with sarin spontaneously reactivated very rapidly in vitro and in vivo with half-times of 18 and 120 min, respectively (Bošković et al., 1984; Clement, 1984; Jokanović, 1989, 2009b; Jokanović et al., 1996).

Various OPs inhibit both CarbE and AChE at similar concentrations ranging from 1 to 1000 nmol/L. CBDP, dichlorvos, DFP, and paraoxon show higher affinity toward CarbE in vitro and as a result their acute toxicity is lower in contrast to highly toxic OPs, soman and sarin, that have four to six times higher affinity for AChE. This relationship was confirmed in vivo after administration of 0.9 LD_{50} of these compounds (Maxwell, 1992). Rat plasma CarbE appears to be more sensitive for soman and sarin than CarbE in rat liver and brain, and can be completely inhibited at sublethal doses. Significant inhibition of CarbE in liver can be obtained only at multiple lethal doses (Bošković et al., 1984). Even when two-thirds of rat liver was removed by partial hepatectomy, 5 LD_{50} of soman was not sufficient to achieve significant inhibition of rat liver CarbE (Jokanović, 1990). Somani et al. (1992) found that interspecies variation in response to some nerve agents may be accounted for largely by CarbE binding.

In a study of the mechanism of interaction of CarbE with some OPs in vitro it was found that this reaction is not irreversible, but reversible due to rapid spontaneous reactivation of inhibited CarbE (Jokanović et al., 1996; Jokanović, 2001, 2009b). The highest rate of spontaneous reactivation was obtained for plasma CarbE inhibited with sarin and the half time of reactivation was 18 min. These results were also confirmed in experiments in vivo in which rats were treated with 0.5 LD_{50} of soman, sarin, and dichlorvos (Jokanović et al., 1996). Calculated half-times of reactivation for plasma CarbE of the rats treated with 0.5 LD_{50} dichlorvos, sarin, and soman were 1.2, 2.0,

and 2.7 h, respectively. Similar results were reported by Gupta et al. (1987a) who found 50% of spontaneous reactivation of plasma CarbE 24 h after poisoning of rats with 100 μg/kg soman. Gupta et al. (1987b) also reported 94% of reactivation of plasma CarbE in rats treated with 200 μg/kg tabun, but 7 days after poisoning. Human CarbE1 reactivated spontaneously after phosphorylation by the most toxic P_s stereoisomer of sarin but not soman or cyclosarin (Hemmert et al., 2010). Spontaneous reactivation of CarbE hydrolyzing phenyl valerate inhibited with paraoxon in vitro was observed by Barril et al. (1999).

55.3.2.4 The role of CarbE in detoxification of OP

CarbEs participate in detoxification in three different ways. The first is hydrolysis of ester bonds in OPs that contain them, such as malathion (World Health Organization, 1986; Fukuto, 1990). The second is binding of OPs to CarbE and other proteins, which decreases the concentration of free OP in the circulation that can react with AChE in vital tissues (Clement, 1984; Jokanović, 1989, 2009b). The third role is related to all OPs that can phosphorylate CarbE by binding to serine hydroxyl group at its active site (Jokanović et al., 1996; Jokanović, 2001, 2009b, 2015b). During spontaneous reactivation this phosphoryl residue is separated from the enzyme-accepting hydroxyl group from water as its new acyl radical. This newly formed OP (i.e., organophosphoric acid) is a much less potent, if at all, esterase inhibitor that represents nontoxic metabolite of the parent OP. In the case of nerve agents the corresponding metabolites formed EDMPA (for tabun), IMPA (for sarin), and PMPA (for soman) are shown in Fig. 55.1. CarbE activity recovered in this reaction can be inhibited again by other OP molecules. The active role of CarbE in this process is in its involvement in metabolic transformation of OP to its nontoxic and biologically inactive metabolites. Because of the rapid spontaneous reactivation of CarbE, one active site at the enzyme can metabolize numerous OP molecules and this reaction does not occur according to the stoichiometric ratio 1:1, depending only on the stability of the bond between phosphorus from OP and oxygen from the serine hydroxyl group. Tissues in which this "turnover" is rapid, such as plasma, have a higher capacity for detoxification of OPs than expected only on the basis of the catalytic activity of CarbE. This reaction can be very important under conditions of repeated (subchronic or chronic) exposure to low doses of nerve agents and other OP that could be inactivated through the reaction with CarbE without any apparent toxic effect (Jokanović, 2015b).

The role of CarbE as a bioscavanger involved in detoxification of nerve agents and other OPs was investigated. The ideal OP bioscavanger would have a fast rate of reactivity for a broad range of OP compounds, a slow rate of aging, and the ability to reactivate to increase its stoichiometry as a bioscavanger. Evaluation of CarbE on these criteria suggests that it is an important candidate as an OP bioscavanger (Doctor et al., 2001). One of the most important advantages of CarbE is that OP-inhibited CarbE does not undergo the rapid aging that prevents oxime-induced reactivation of OP-inhibited cholinesterases. This means that OP-inhibited CarbE can be reactivated yielding an active enzyme, involved in further metabolism of OP molecules, and an inactive OP metabolite (Jokanović, 2015b).

Another advantage of CarbE is the much greater size of its active site compared to AChE (10-fold difference) and ChE (sixfold difference) (Saxena et al., 1999). The large active site volume of CarbE minimizes steric hindrance effects at the active site and maximizes the potential for reactivation. In a study investigating the structural specificity of AChE, ChE, and CarbE, Maxwell et al. (1998) reported that AChE could accommodate OP inhibitors containing only one bulky group (e.g., isopropyl, pinacolyl, phenyl), ChE could accommodate OP inhibitors containing two of the smaller bulky groups (like isopropyl), while the active site of CarbE was sufficiently large to accommodate up to two of the largest bulky groups (e.g., phenyl groups). Therefore, CarbE had the ability to metabolize the broad spectrum of OP inhibitors. The only exception to this observation is, due to fewer aromatic residues in the active site of CarbE in comparison to ChE, reduced affinity of CarbE for positively charged OP inhibitors (such as VX) and this effect apparently has little importance for nerve agents (except VX) and OP pesticides since a few of them are positively charged (Doctor et al., 2001).

55.3.2.5 Prolidase (EC 3.4.13.9)

There are data in the literature showing that prolidase (EC 3.4.13.9), a naturally occurring enzyme of mammalian and bacterial origin, is involved in hydrolysis of G-type nerve agents. Recombinant HuPON1 and DFPase showed 10-fold lower activity toward sarin compared to recombinant human prolidase (Cheng et al., 1999; diTargiani et al., 2010; Nachon et al., 2013). Constante et al. (2012) reported that partially purified human liver prolidase hydrolyzed DFP and various nerve agents, while skin and kidney prolidase presented significantly lower activity against soman, tabun, and VX. The biochemical characteristics of prolidase purified from human erythrocytes, liver, kidney, and fibroblast cells are well known. However, OP hydrolyzing activity of prolidase is still not well understood (Wang et al., 2006; diTargiani et al., 2010; Chandrasekaran et al., 2013).

Human prolidase is a 54-kDa binuclear Mn^{2+}-dependent enzyme which breaks the amide bond in dipeptides containing proline or hydroxyproline as the C-terminal amino acid. It plays a crucial role in the recycling of proline. Prolidase is found in most tissues and in several animal species. Deficiency of this enzyme in humans results in a syndrome with a highly variable clinical phenotype, such as chronic recurrent infections, mental retardation, splenomegaly, skin lesions, and the excretion of massive amounts of iminopeptides in urine (Wang et al., 2006; Chandrasekaran et al., 2013).

55.4 Lipase

Touvrey et al. (2019) identified human bile salt-activated lipase, also denoted as pancreatic lipase, as a potential target for OP. They determined bimolecular inhibition rate constants for the reaction of the enzyme with sarin, tabun, VX, their surrogates, and paraoxon. Finally, they determined different OP-inhibited X-ray structures that would help the design of variants with potential OP-hydrolyzing properties. This finding appears to be the first time that the interaction of several OPs with a lipase has been discussed. Interaction of OP with the lipase could serve as another detoxification mechanism, in addition to OP reaction with serum ChE, CarbE, and PON, that can decrease the amount of free OP in the circulation available for AChE inhibition (Jokanović, 2019).

55.5 Protein binding

Proteins are amphotheric structures containing anionic and cationic reactive sites. Proteins can also participate in other interactions with xenobiotics through the formation of hydrogen bonds, polarity, electrostatic, and Van der Waals forces. Many xenobiotics can bind to proteins from blood such as albumin and B-esterases. Easy binding to proteins occurs with substances that are ionized at physiological pH and those soluble in lipids such as OP. After binding of OP to proteins such as CarbE, ChE, AChE, and other macromolecules, these agents are metabolized since their acyl radical is released and phosphoryl residue remains bound to proteins. This unspecific binding of OP to blood proteins decreases the OP concentration in the circulation and tissues, preserving AChE activity at target sites (Jokanović, 2009b). Binding of OP to proteins can be limited by steric hindrance, protein conformation factors that do not allow OP molecules to access all binding sites at the protein. The involvement of secondary noncholinergic targets (neuropathy target esterase, fatty acid amide hydrolase, arylformamidase, acylpeptide hydrolase, and other macromolecules) in OP toxicity was reviewed by Casida and Quistad (2004).

Albumin is the most abundant protein found in plasma, having a half-life of about 20 days. It is a 67-kDa multifunctional monomer synthesized and secreted by the liver. Albumin has esterase-like and aryl acylamidase activities but it does not have a catalytic active-site serine (Marsillach et al., 2013). The high concentration of albumin in plasma (30−60 g/L) may balance the poor reactivity of this protein with OPs (Jokanović, 2009b, 2015b).

Williams et al. (2007) found that sarin, soman, cyclosarin, and tabun phosphorylate a tyrosine residue on albumin in human blood. The tyrosine adducts with soman and tabun were detected in guinea pigs receiving therapy 7 days following subcutaneous administration of 5 LD_{50} of the respective nerve agent. Bao et al. (2012) reported formation of soman, sarin, and VX adducts with the tyrosine residue of albumin when rats were exposed to sublethal doses of the nerve agents. In blood samples taken from sarin-, soman-, cyclosarin-, or tabun-treated marmosets, tyrosine adducts were detected after 23 or 24 days (Read et al., 2010). Li et al. (2008) have shown that soman covalently binds to albumin at tyrosine 411 and that the adduct was stable ($t_{½} = 20$ days). However, although the concentration of albumin in plasma is very high, its reactivity with soman was apparently too slow to play a major role in detoxification of the agent. The authors concluded that soman−albumin adducts could be useful for the diagnosis of soman exposure. In addition, OP pesticides covalently bind to albumin and the adduct was stable for more than 7 days (Tarhoni et al., 2008).

Tyrosine adducts were found in guinea pigs and marmosets poisoned with sarin, soman, cyclosarin, and tabun. VX, being less reactive than other WNA, formed an adduct in human plasma in vitro only at high concentrations (Williams et al., 2007; Black, 2010; Black and Read, 2013). Tyrosine adducts were less sensitive than ChE as biomarkers with respect to exposure levels, but were more stable and did not undergo an aging reaction like OP bound to serine esterases (Read et al., 2010). However, albumin might have a larger capacity for OP binding compared to ChE and other esterases.

55.6 Concluding remarks and future directions

After more than five decades of research into the biotransformation of nerve agents soman, sarin, tabun, and VX it can be concluded that several enzymes have a significant role in this process. Enzymes capable of hydrolyzing these agents (A-esterase, PON1) were very efficient in breaking down the bond between phosphorus and acyl radical and their activity was proportional to substrate concentration. Esterases such as cholinesterases and CarbEs act by binding of OP molecules to the hydroxyl

group placed in their active site, decreasing free concentration of the agents in blood, thus preventing inhibition of acetylcholinesterase at target sites and subsequently their toxic effects. In addition, binding of OP to CarbEs is reversible, indicating an active role of the enzyme in metabolic detoxification of nerve agents and other OPs. The importance of prolidase and lipase is still unclear and requires further studies. It is necessary to further investigate the role of these enzymes and other macromolecules in detoxification of OP compounds and their possible application in prophylaxis and treatment of OP poisoning in humans.

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Chapter 56

Laboratory analysis of chemical warfare agents, adducts, and metabolites in biomedical samples

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56.1 Introduction

Chemical warfare agents (CWAs) are the most toxic compounds ever produced. The need for the analysis of biomedical samples can have several purposes. First, the agents themselves may be detected in their intact form in the case of toxicokinetic studies, which is essential for a better understanding of the toxicological processes. Toxicokinetic studies provide a quantitative basis for the development of new strategies for prophylaxis and therapy against intoxication with CWAs. Second, verification of exposure to CWAs is another goal that requires analytical methodology for biomedical samples. Verification of exposure is needed for several reasons:

1. In the case of a CWA exposure, military personnel need a rapid diagnosis to ascertain the level of exposure and the identity of the agent, in order to give victims adequate medical treatment.
2. Low-level exposures to nerve agents might be associated with unexplainable phenomena such as the Gulf War syndrome (Central Intelligence Agency, 1997).
3. In the case of a terrorist event, or other cases of alleged use, unambiguous verification is needed to verify the exposure. Analyses of biomedical samples can also be used as evidence in a court of justice or international community (United Nations, 1986). In the summer of 2013, the OPCW, commissioned by the United Nations, collected environmental and biomedical samples in Syria (UN Report, 2013). The analysis of the biomedical samples provided evidence that sarin was used in the city of Saraqueb, Syria, in April 2013 (John et al., 2018). The events in Syria revealed that biomedical samples are very important. In a case that victims show clear symptoms of poisoning, it provides a clear direction for sampling. In the case of environmental sampling, these indications are often less clear and, moreover, often it is not possible to enter the affected (or occupied) area. On the other hand, victims can often reach the forensic team on their own or they are brought to the team. Ultimately, in the case of a small-scale attack, the biomedical samples of a single intoxicated person might be the only indication. Methodologies for the verification of exposure to chemical warfare agents have been published in special issues of the *Journal of Analytical Toxicology* (Barr, 2004, 2008; Black, 2008). The methodology of the clinical methods has also been reviewed by Noort et al. (2002), Noort and Black (2005), Black and Noort (2005, 2007), and Capacio et al. (2008a,b, 2019). This chapter is not meant as a duplicate for these references. It will discuss the updated analytical methods that are needed for the analysis of biomedical samples. Most of the analytical methods rely on chromatographic techniques like gas and liquid chromatography. The progress in instrument development on the detection site has been tremendous in the last few decades. Analyses based on mass spectrometry are now more or less routine. Triple quadrupole and high-resolution mass spectrometers have become affordable and automated standard configurations with mass spectrometers now fulfilling the analytical needs for most types of analyses. Analyses of chemical warfare agents themselves might need some additional requirements. For example, it might be necessary to measure an intact agent at extremely low concentrations, because only these levels are relevant in view of the high toxicity of the agents.

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In that case, it might be considered to utilize large-volume sample introduction. This puts higher demands on the analytical configurations because the introduction of larger sample volumes also increases the matrix effect, which puts extra demands on the selectivity of the analysis. In the special case of nerve agents, it might be desirable to distinguish the stereoisomers of these compounds from each other, which requires a rather complicated analytical configuration. In this chapter the methods for the bioanalysis of CWAs or their biomarkers are briefly described and in the case that the instrumentation for a particular analysis is more sophisticated than a standard configuration, it is discussed in more detail.

56.2 Nerve agents

56.2.1 Analysis of intact nerve agents

Nerve agents are organophosphorus (OP) compounds that rapidly inhibit acetylcholinesterase (AChE), which results in an accumulation of acetylcholine (ACh), leading to muscle fasciculations and paralysis, and finally resulting in death (Dacre, 1984). For toxicokinetic studies it is required to analyze the intact agent. The toxicokinetic studies create insight into the distribution and elimination of the agents and indicate until which time toxicologically relevant concentrations are still present in the circulation (Benschop and De Jong, 2001; Van der Schans et al., 2008b). In view of the high toxicity of nerve agents, these toxicologically relevant concentrations are in the pg/mL range. Using gas chromatography, these levels can only be measured when large-volume samples can be introduced into the column. With higher sample volumes, the matrix effect also is increased, and it puts higher requirements to the selectivity of the analysis. Another challenging factor is that nerve agents are chiral compounds with an asymmetrical phosphorus atom (Benschop, 1975; Benschop and De Jong, 1988). The difference in toxicity of the two isomers is several orders of magnitude, the P (−)-isomers being the most toxic (Benschop et al., 1981, 1984; Benschop and De Jong, 1988). In the case of toxicokinetic studies, it is important to differentiate between the two isomers because it is essential to know which isomer is still present in the circulation. Chiral gas chromatography can fulfill this requirement for G-agents like sarin and soman. Soman has two asymmetric atoms, phosphorus and carbon, in the pinacolyl group. Therefore, the compound consists of four different stereoisomers. An optical active stationary phase like beta-cyclodextrin can be used to separate the isomers of sarin (Spruit et al., 2001). Chirasil-Val columns can be used to separate the four isomers of soman (Benschop and De Jong, 1991; Benschop et al., 1981, 1985). The Chirasil-Val stationary phases were synthesized at the TNO Prins Maurits Laboratory. Smith and Schlager (1996) and Yeung et al. (2007) reported the use of commercially available gamma-cyclodextrin-based columns that are also capable of separating the four isomers of soman.

In the case of toxicokinetic studies the chiral columns were installed in a two-dimensional GC configuration according to the heart cutting method. First, the introduction of large-volume samples requires additional selectivity from the chromatographic system to alleviate the matrix effect. Second, the fragile optical active phase needs to be protected from the "dirty" extracts of the biosamples and the condensating solvent in the column. Samples are introduced by thermodesorption tubes filled with Tenax absorption material. The components are cryo-focused in a cold trap and after a certain desorption time, flash injected into the first column. According to the heart cutting technique, one small section of the effluent of the first column is collected in a cold trap and then re-injected on the second analytical column with the chiral selective phase (Due et al., 1993). The isomers of soman can be detected in the blood of guinea pigs at levels down to10 pg/mL blood (Benschop and de Jong, 1991).

The chiral separation of VX isomers was achieved using normal-phase liquid chromatography with a Chiracel OD-H column. The separation of the enantiomers of VX was first described by Kientz et al. (1994). They used a thermo-ionic detector for the selective detection of phosphorus compounds. Unfortunately, this technique was not robust enough. The same separation was also described by Van der Schans et al. (2003) using electrochemical detection. The system could be used to study the stereo-selective degradation of VX in in vitro samples such as liver homogenates and plasma. Although the electrochemical detection was rather sensitive (absolute detection limit 25 pg), the method was not selective enough to measure low levels of VX in extracts of biological matrices at a relevant level (<10 ng/mL). VX can be relatively easily extracted from blood or plasma using organic extraction with hexane (Bonierbale et al., 1996). Relevant levels of VX in blood could be detected down to 1 ng/mL using a two-dimensional offline LC−GC separation (Van der Schans et al., 2003). Smith (2004) published the same separation using a single quadrupole mass spectrometer using atmospheric pressure chemical ionization (APCI). In that paper, he focused on the separation and detection of VX to study the stereo-selective degradation of VX in plasma. Reiter et al. (2008) published the chiral separation of VX isomers with gas chromatography using a HYDRODEX-β -TBDAc column. In the same article, the authors reported also the chiral separation of VX isomers using LC-MS with a Chiral AGP column. Using this analytical method, they were able to study the percutaneous toxicokinetics of VX in pigs Reiter et al. (2008).

56.2.2 Verification of exposure to nerve agents

There are several strategies available to verify an exposure to nerve agents. It is not feasible to measure the intact reagent, since the half-life of these agents is only a few hours, which means that they disappear within a day after exposure (Benschop and de Jong, 2001; Van der Schans et al., 2008b, 2019). Metabolites, often alkylmethylphosphonic acids, are better biomarkers because they circulate for a longer period of time and are gradually excreted in urine (Shih et al., 1994; Fredriksson et al., 1995; Nagao et al., 1997a; Nakajima et al., 1998; Noort et al., 1998; Barr et al., 2004). Several methodologies have been published to analyze these metabolites, which were found in plasma and urine. The alkylmethylphosphonic acids can be isolated from plasma or urine using liquid−liquid extraction (Noort et al., 1998; Katagi et al., 1997; Barr et al., 2004) or solid-phase extraction (Hamelin et al., 2014). Most methods for determination of these compounds are based on liquid chromatography or gas chromatography, which requires derivatization (Black and Muir, 2003). Selective tandem MS techniques facilitated by triple quad instruments or high-resolution MS instruments enabled the detection of hydrolysis products in plasma and urine down to the pg/mL range (Driskell et al., 2002; Barr et al., 2004; Riches et al., 2005; Evans et al., 2008; Swaim et al., 2008; Hamelin et al., 2013, 2014). This concentration is so low that hydrolysis products can be detected in urine up to 1 week after exposure (Riches et al., 2005). The hydrolysis product of sarin was also detected in samples from victims of the attack with sarin in Tokyo (Nagao et al., 1997b; Matsuda et al., 1998) and from victims of the attack with sarin in Syria (John et al., 2018).

Nerve agents bind to proteins such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). These proteins are not excreted or metabolized rapidly (typical half-life is 12 days for BuChE; Hall et al., 1984), which means that adducts to proteins can serve as retrospective biomarkers for exposure to nerve agents (Polhuijs et al., 1997; Black et al., 1999; Fidder et al., 2002; Van der Schans et al., 2004a). The enzymatic measurement of AChE activity, known as the Ellman assay, is the easiest method to determine a nerve agent exposure (Ellman et al., 1961; Halbrook et al., 1992). The method is based on the enzymatic cleavage of the substrate acetylthiocholine where after thiocholine reacts with 5,5′-dithiobis-(2-nitrobenzoic acid), which forms 2-nitro-5-thiobenzoate, a yellowish product that can easily be measured with a low-cost colorimeter. The method has been optimized for high-throughput analysis (Doctor et al., 1987; Halbrook et al., 1992; Worek et al., 1999; Eyer et al., 2003) and is also available in fieldable kits (Taylor et al., 2003). Major drawbacks of the method are, first, that the identity of the nerve agents cannot be elucidated from this measurement. Second, the intra- and interpersonal variation of the ChE activity implies that a decrease in ChE activity must be relatively large to be significant (Brock, 1991; Lotti, 1995; Wilson et al., 1997; Nigg and Knaak, 2000). Third, the de novo synthesis of the enzyme restores the enzyme activity within several days to within the range of the control values. Low-level exposures to nerve agents or exposures that took place several weeks before the biosample could be taken cannot be detected using this method. Therefore, instead of looking for a decrease in AChE activity, it is more efficient to detect the ChE fraction that is inhibited by the nerve agent. In that case, it is also better to look at BuChE, which has several advantages over AChE. First, BuChE is a protein present in plasma, which is an easier sample matrix to process than whole blood. Second, the concentration of BuChE is approximately 80 nM, which is approximately 10 times higher than the concentration of AChE in blood (Myers, 1952; De Jong and Wolring, 1984). This automatically means that the concentration of that biomarker is higher and therefore easier to detect. The most straightforward method to detect the adduct of nerve agent is the fluoride reactivation method (Heilbronn, 1964, 1965; De Jong and Van Dijk, 1984; Polhuijs et al., 1997). During incubation of a plasma sample with fluoride ions, the nerve agent adduct is released from the protein and can be extracted in a GC-compatible solvent and subsequently analyzed with GC. The lowest detectable degree of inhibition that can be determined with this method depends on the type of GC detector. Typical reasonably priced detectors like the nitrogen phosphorus detector (NPD), flame photometric detector (FPD), and mass selective detector show absolute detection limits of about 1 pg, which means that a concentration of 1 ng/mL can be detected using an injection volume of 1 μL. A concentration of 1 ng/mL of nerve agent is equivalent to 5−7 nM, which corresponds with approximately 10% BuChE inhibition. In order to be able to determine lower degrees of inhibition, higher sample volumes have to be injected, which puts higher demands on the selectivity of the separation and detection method. Another option is to improve the selectivity of the detector. Chemical ionization with ammonia as a reaction gas is a relatively soft ionization mode, which ensures a more selective detection of only compounds with sufficient proton affinity (Degenhardt et al., 2004; Jakubowski et al., 2004). Another more expensive but recently affordable option, is to use a high -resolution MS or tandem MS instrument, which can provide sensitivity and selectivity for the sensitive detection of low-degree inhibition (Degenhardt et al., 2004; Lee and Lee, 2014). When the large-volume technique or the sample has been preconcentrated by evaporating the solvent, low degrees of inhibition down to 0.1% can be confirmed (Holland et al., 2008).

The method has been used to analyze samples from victims of the sarin attack in the Tokyo subway (Polhuijs et al,1997) and samples that were taken from victims that were exposed to sarin in Syria in 2013 (John et al., 2018). The method has also been used to verify exposure to VX in accidental exposures (Solano et al., 2008). The method is also used in toxicological studies to determine the distribution of nerve agents in tissues (Adams et al., 2004). The persistence of the biomarkers was also studied in rhesus monkeys that were injected with a sign-free dose of nerve agent. Regenerated sarin could be detected up to 54 days after exposure (Van der Schans et al., 2004a).

A major drawback of the fluoride reactivation method is that not all nerve agent adducts are amenable to fluoride reactivation, with the aged adduct of soman the best known example. This problem can be solved by looking at the BuChE enzyme itself. Fidder et al. (2002) published a method based on the LC−MS/MS analysis of a nerve agent phosphorylated nonapeptide (FGES*AGAAS) derived after pepsin digestion of inhibited butyrylcholinesterase (BuChE). The phosphorylated nonapeptide is best analyzed with an LC tandem MS instrument using the single reaction monitoring (SRM) mode. The mass of the parent ion depends on the mass of the nerve agent that is conjugated to the peptide. During the fragmentation process, the phosphyl moiety of the nerve agent is removed first and the characteristic daughter ions are *m/z* 778, 673, and 602, which are all fragments of the native peptide. Fig. 56.1 shows the mass spectrum of the nonapeptide with methylphosphonic acid adduct, resulting after exposure to a rapidly aging nerve agent such as soman. This mass spectrum has been acquired on a QTOF instrument with accurate mass determination. The spectrum shows that the mass difference between the protonated parent ion and the highest fragment corresponds with the theoretical mass of methylphosphonic acid. With this result, high-resolution mass spectrometers show their added value in unambiguous identification of CWA adducts. Product ions of the phosphorylated nonapeptide could successfully be identified within 5 ppm deviation from their chemical formulas. The analysis of the phosphorylated nonapeptide was successfully applied on plasma samples that were taken from victims who had been exposed to sarin in the Tokyo subway in 1995 (Polhuijs et al., 1997; Fidder et al., 2002) and from victims of the attack with sarin in Syria in 2013 (John et al., 2018). Fidder et al. (2002) used a procedure to extract BuChE from plasma using home-fabricated procainamide gels. In the last couple of years the sample preparation procedure for the isolation of BuChE from plasma has been further improved. Sporty et al. (2010) described the use of antibody-loaded magnetic beads to isolate BuChE from plasma, which resulted in a better sample clean-up and better possibilities for automation of the method. Carter et al. (2013) optimized the method further for quantitation of the nerve agent adduct and Knaack et al. (2012) developed a method enabling high throughput of samples. Schopfer et al. (2019) reported the use of Hupresin affinity chromatography to purify BuChE as an alternative for the isolation of BuChE using antibodies. The analysis of FGESAGAAS nonapeptide adducts is nowadays also used for biomonitoring for exposure to other cholinesterase inhibitors. For example, Schopfer et al. (2010) and Johnson et al. (2015) used the same peptide for diagnosis of 2-(ortho-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP) exposure.

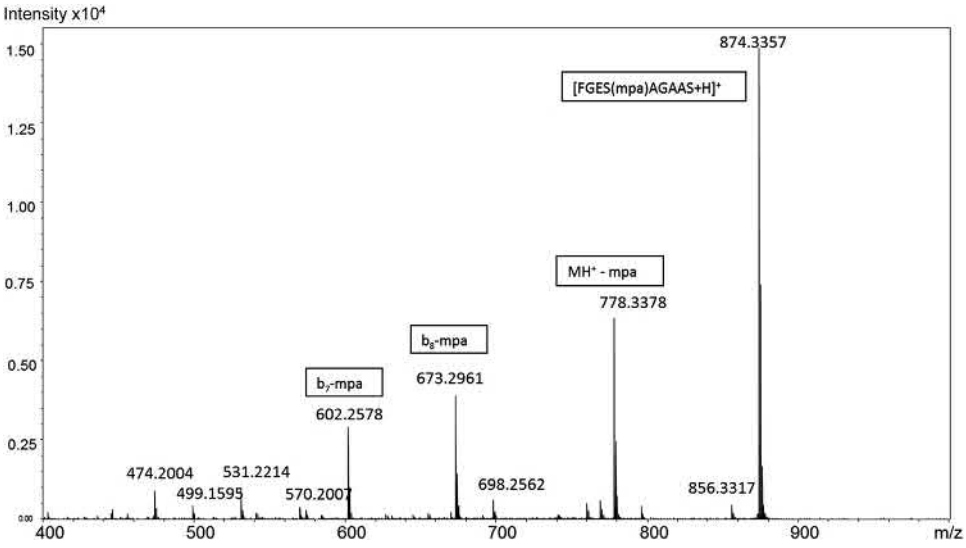

FIGURE 56.1 MS-MS chromatogram of protonated ion $[MH]^+$, *m/z* = 874.3357 representing the nonapeptide FGESAGAAS with methylphosphonic acid adduct. The mass difference between $[MH]^+$ and "$[MH]^+$-mpa" (*m/z* 95.9979) is identical to the theoretical mass of methylphosphonic acid (*m/z* 95.9976).

Due to the low concentration of the biomarkers, the analyses are target directed because a full scan would be at the cost of sensitivity, which means that low-level exposures cannot be detected. However, this is a serious problem in the case where the identity of the nerve agent is not known beforehand. Some progress has been achieved to solve this problem. The number of different OPs exceeds several thousand, but the number of different masses is only 170. Since the OPCW Schedule 1 nerve agents consist only of saturated alkyl groups with mass increments of 14 units the number of mass possibilities can be further reduced to 36 masses, which means that only 36 MRM transitions have to be recorded (Van der Schans et al., 2008a). It is anticipated that the newest mass spectrometers will be able to acquire all MRM transitions, relevant to all nerve agents. Another approach was published by Noort et al. (2006). The phosphorylated serine residue of nerve agent-inhibited BuChE can be converted under alkaline conditions into a dehydroalanine residue that can subsequently react with a generic tag, yielding a mutual product, whatever the identity of the nerve agent. The resulting product can be detected in the most sensitive single reaction monitoring mode of the mass spectrometer.

In addition to BuChE, albumin is also a target protein for nerve agents, especially at Tyr^{411}, be it with a lower affinity than BuChE. After digestion with pronase, the phosphorylated tyrosine can be detected with LC-MS. Therefore, nerve agent adducts to albumin are also a valuable biomarker for nerve agent and pesticide exposure (Black et al., 1999; Williams et al., 2007; Read et al., 2010; Van der Schans et al., 2013; Crow et al., 2014).

In the case of an intoxication with VX, the leaving group upon binding to proteins is diisopropylethane-thiol. The compound is that specific for VX that it can serve as a representative biomarker of exposure of VX. The compound circulates in plasma and can be analyzed with GC after extraction with an organic solvent (Bonierbale et al., 1996). The free thiol group is amenable to react with proteins such as albumin where it will bind with various Cys residues to form a disulfide bond. Kranawetvogl et al. (2017, 2018) reported the analysis of a Cys*-Pro dipeptide and a Met-Pro-Cys* tripeptide derived after digestion with pronase, being the most prominent additional biomarker of exposure to VX.

56.3 Sulfur mustard and lewisite

There is a variety of biomarkers that can verify an exposure to sulfur mustard. Analogous to the nerve agents, biomarkers can be distinguished in metabolites that are excreted in urine and adducts to proteins. Thiodiglycol (TDG) and its oxidation product thiodiglycol sulfoxide (TDG-sulfoxide), are the oldest biomarkers of exposure to sulfur mustard (Black and Read, 1988, 1991). The compounds can be isolated from urine or plasma using solid-phase extraction and detected with GC-MS. Wils et al. (1985, 1988) treated TDG with hydrochloric acid to convert TDG back into sulfur mustard. Black and Read (1991) reported a method that detects TDG, TDG-sulfoxide, and their acid-labile esters, as the single analyte thiodiglycol. In both cases the recovered analytes were converted to the bis(pentafluorobenzoyl) derivative of thiodiglycol and detected by GC-MS using negative ion chemical ionization. The limits of detection were 1 ng per 0.5-mL sample of urine. Later the sensitivity of the method was further improved using GC-tandem MS (Riches et al., 2007). The biomarker has been detected in the urine of victims that were exposed to sulfur mustard in the Iran−Iraq War in the 1980s (Wils et al., 1985, 1988) and later in urine of persons that were accidently exposed to sulfur mustard (Jakubowski et al., 2000).

The drawback of these TDG and TDG-sulfoxide as biomarkers is that they suffer from high background levels. In that respect, a series of more specific metabolites were discovered by Black et al. (1991). Sulfur mustard reacts with glutathione, which is then further metabolized by the β-lyase enzyme. Two β-lyase metabolites have been identified in the urine from exposed individuals: 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl] ethane (MSMTESE) and 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE). MSMTESE and SBMSE can be reduced using $TiCl_3$ and analyzed by GC/MS/MS as a single analyte: 1,1.-sulfonylbis[2-(methylthio)ethane] (SBMTE) (Black et al., 1991; Black and Read, 1995a,b; Young et al., 2004; Daly et al., 2007). MSMTESE and SBMSE can also be analyzed individually without reducing the two analytes to a common analyte using electrospray LC-MS/MS (Read and Black, 2004a,b; Halme et al., 2011; Li et al., 2013; Liu et al., 2017). To date, no background levels of SBMTE have been found in the urine of unexposed individuals, including studies where urine samples from over 100 individuals were analyzed using two different assay methods (Boyer et al., 2004; Young et al., 2004). These metabolites have been measured in persons that were accidently exposed but were also analyzed in scientific animal experiments in order to study the toxicology of the agent and the persistence of the biomarker in the circulation (Black et al., 1992a,b; Barr et al., 2005, 2008; Lin et al., 2014).

Analogous to nerve agents, sulfur mustard binds to proteins and the protein adducts are a potential biomarker for exposure to sulfur mustard (Black et al., 1997a,b). The sulfur mustard adduct to N-terminal valine of globin in blood can be analyzed after a sample preparation procedure known as the modified Edman degradation (Fidder et al., 1996a,b; Noort et al., 1996, 1997, 2000, 2004a,b). The lower limit of detection for the assay was determined

using in vitro exposures of sulfur mustard in human whole blood and was determined to be equivalent to a 100 nM exposure level (Fidder et al., 1996a,b; Noort et al., 2004a). The method has been applied to samples from victims that were exposed to sulfur mustard in the Iran−Iraq War in the 1980s (Benschop et al., 1997). The persistence of the biomarker was also studied in marmosets that received a dose of 4.1 mg/kg i.v. The valine adduct could be detected up to 94 days after exposure (Noort et al., 2002).

Sulfur mustard binds also to cysteine in albumin and this adduct can be analyzed as a tripeptide Cys*-Pro-Phe, where sulfur mustard binds to the cysteine residue. The modified tripeptide can be detected after isolating albumin using affinity chromatography, enzyme digestion with pronase, and analysis of the alkylated peptide fragment using LC-tandem mass spectrometry. Exposure levels down to 10 nM could be detected using this method (Noort et al., 1999, 2000, 2004b; Liu et al., 2015). In a retrospective study, rats were injected with a dose of 0.3 mg/kg. The modified tripeptide could be detected up to 7 days after the injection (Noort et al., 2008). The adduct to the *N*-terminal valine in hemoglobin could still be detected after 28 days.

Several other authors published various methods on the same biomarker. Pantazides et al. (2015) treated the precipitated plasma proteins with proteinase K, followed by further sample cleanup using solid-phase extraction and analysis with LC-tandem MS. Andacht et al. (2014) ruggedized the method further to enable enhanced throughput for quantification of sulfur mustard adducts. The same method can also be used for the detection of nitrogen mustard adducts (Pantazides et al., 2019). The alkylated tripeptide is one of the most important biomarkers and has been measured in plasma samples from accidently exposed persons (Smith et al., 2008). Further modifications to the method detecting the same adduct site but detecting as a shorter peptide, HETE-Cys-Phe, have also been published (Gandor et al., 2015; John et al., 2016). The methods have also been used for the forensic analysis of samples obtained from victims that were exposed to sulfur mustard in a crisis region in the Middle East in 2015 (John et al., 2019).

The histidine residue in the globin protein is another target for binding of sulfur mustard (Black et al., 1997b). Following digestion with pronase, the sulfur mustard histidine analyte (HETE-His) could be detected with LC tandem MS after exposure of human blood at an exposure level of 10 μM sulfur mustard in vitro (Noort et al., 1997).

Analogous to the fluoride reactivation method for nerve agents, the sulfur mustard adduct can also be released from the protein by alkaline hydrolysis and is measured as thiodiglycol. A precipitation step during the sample preparation removes endogenous thiodiglycol before it is released from the protein (Capacio et al., 2004, 2008a,b; Lawrence et al., 2008). Unlike the nerve agents, the analyses can be target directed and the mass spectrometer can be operated in the most sensitive SIM or SRM mode.

Sulfur mustard, being a strong alkylating agent, can also react with DNA. In particular, the guanine base is susceptible for alkylation resulting in the formation of *N*7-[2-[(2-hydroxyethyl)thio]-ethyl]guanine adduct. Wei et al. (2011) reported an analytical method based on the digestion of DNA from exposed whole blood followed by analysis with LC-tandem MS. The sulfur mustard guanine adduct is also excreted in urine and Fidder et al. (1996a,b) published a method using GC-MS or LC-MS/MS. Recently, sulfur mustard adducts to the other bases of DNA have also been discovered (Yue et al., 2014), but the relevance of this biomarker in real cases is still under investigation (Zubel et al., 2017, 2019). In addition to the methods based on instrumental analysis, sulfur mustard adducts to DNA can also be detected using immunoassays. Antibodies that can recognize the sulfur mustard adducts have been raised. In an immunoslotblot assay, exposures to sulfur mustard could be verified in DNA obtained from blood or skin (Benschop et al., 1997; Van der Schans et al., 1994, 2002, 2004b).

In the case of toxicokinetic studies of sulfur mustard, typical concentrations at 10−100 pg/mL in blood were found and could not be measured with a normal GC−MS configuration. The utilization of large-volume sample introduction by thermodesorption and two-dimensional chromatography (analog to the configuration for chiral nerve agent analysis) enabled the detection of sulfur mustard levels down to 10 pg/mL (Oostdijk et al., 2007). The two-dimensional chromatography was necessary at that time to ensure sufficient selectivity. Nowadays these studies could be performed using GC-MS/MS systems operating in SRM mode that also ensures sufficient selectivity (Nawała et al., 2016). Langenberg et al. (2016) described toxicokinetic studies of sulfur mustard that were performed using two-dimensional chromatography coupled with MS.

The number of methods to verify an exposure to lewisite is limited. Chlorovinylarsonous acid (CVAA) can be found as the main metabolite in urine. Wooten et al. (2002) developed a simple assay based on solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) after derivatization of CVAA with 1,3-propanedithiol (PDT). Fidder et al. (2000) developed a method based on extraction of bound and unbound 2-chlorovinylarsonous acid (CVAA), a major metabolite of L1, from whole blood after treatment with 2,3-dimercapto-1-propanol (BAL). Subsequent to derivatization with heptafluorobutyryl imidazole, the CVAA-BAL

derivative could be analyzed at a 40-fmol level by means of gas chromatography-mass spectroscopy (GC-MS). Using this procedure, in vitro exposure of human blood to 1 nM lewisite could be determined. The same procedure was applied to the analysis of human urine samples spiked with CVAA.

56.4 Concluding remarks and future directions

The development of sophisticated analytical instruments, mainly based on mass spectrometry, enabled several analyses for bioanalysis of chemical warfare agents. Toxicokinetic studies at relevant levels (down to 10 pg/mL blood) could be performed. Exposures to CWAs can be verified up to several weeks after exposure, because of the persistence of the biomarkers and also because of the sensitive and selective instrumentation. It is anticipated that future equipment will be even more sensitive, enabling exposures that occurred weeks after the exposure to be tracked down. Better sensitivity is also desirable, in order to meet the criteria that are in place for the identification of a compound meeting the forensic standards. Meanwhile the OPCW fully recognized the relevance of biomedical samples and implemented a system of biomedical proficiency tests (bioPT). Within the framework of this bioPT, criteria for unambiguous verification of exposure have been defined (Mamidanna, 2016). The criteria dictate that the identification of each test chemical must be based on at least two different analytical methods, or one analytical method for two different biomarkers confirming the exposure of a particular chemical. Sections 56.2 and 56.3 showed that multiple methods are available to verify an exposure. For nerve agents, hydrolysis products can be analyzed with LC-MS or GC-MS. Protein adducts of nerve agents can be divided into BuChE and albumin adducts. For sulfur mustard, urine metabolites such as the β-lyase metabolites can be analyzed. For proteins there is a wide variety of sulfur mustard adducts that can be detected. Tables 56.1 and 56.2 show an overview of the biomarkers that were discussed. Secondly, a total of at least five identification points must be obtained to achieve sufficient analytical data for the identification using a maximum of three techniques. Information-rich techniques such as HR-MS provide more

TABLE 56.1 Major biomarkers for nerve agent exposure.

Matrix	Biomarker	Analyte	Analytical technique
Urine, plasma	Alkylmethylphoshonic acid	Alkylmethylphoshonic acid	LC-MS/MS GC-MS/MS (after derivatization)
Plasma	Adduct to BuChE	Phosphorylated nonapeptide	LC-MS/MS
Plasma	Adduct to various proteins	Regenerated OP- fluoridate	GC-MS/MS
Plasma	Adduct to albumin	Phosphorylated tyrosine	LC-MS/MS

TABLE 56.2 Major biomarkers for sulfur mustard exposure.

Matrix	Biomarker	Analyte	Analytical technique
Urine, plasma	Thiodiglycol	Thiodiglycol	LC-MS/MS, GC-MS/MS (after derivatization)
Urine, plasma	Thiodiglycol-sulfoxide	Thiodiglycol-sulfoxide	LC-MS/MS, GC-MS/MS (after derivatization)
Urine	ß-lyase metabolites, MSMTESE, SBMSE	MSMTESE SBMSE SBMTE (after reduction of MSMTESE and SBMSE with $TiCl_3$)	LC-MS/MS
Plasma	Alkylated globine	HETE-Val	GC-MS/MS (after derivatization)
Plasma	Alkylated globine	N1-HETE-His N3-HETE-His	LC-MS/MS
Plasma	Alkylated albumin	[S-HETE]-Cys-Pro-Phe	LC-MS/MS
Blood	Alkylated DNA	N7-[2-[(2-hydroxyethyl)thio]-ethyl]guanine	LC-MS/MS, immunoslotblot

points (up to four), while low-information techniques provide less points, for example, two points for one transition in an MRM analysis. Mathews et al. (2017) showed that higher confidence in identification is obtained when HR-MS is utilized.

Another interesting development is comprehensive GCxGC. Comprehensive GC offers great selectivity and resolution, ideal for complex samples such as biomedical samples. The combination with time of flight mass spectrometry offers the possibility to analyze in a full-scan mode, not target directed, with the availability of a full mass spectrum that will meet the forensic standards. The utility has been demonstrated for the detection of CWAs in complicated matrices such as fuel (Reichenbach et al., 2003). The method has also been used for the analysis of regenerated sarin in inhibited plasma (Van der Meer et al., 2010). Finally, it may be expected that the sample preparation will be further automated. A promising approach was published by Carol-Visser et al. (2008) and more recently by Bonichon et al. (2018), based on the online digestion and analysis of sulfur mustard adducts on albumin and the sarin adduct to BuChE, respectively.

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Chapter 57

On-site detection of chemical warfare agents

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57.1 Introduction

Chemical warfare agents (CWAs) were employed in World War I and World War II, and during the Cold War, and they continue to be produced and stockpiled even today (Somani, 1992; Gupta, 2015). In the 1980s, Iraq used sarin (GB) and sulfur mustard (HD) in the Iran−Iraq conflict (Black et al., 1994). In 1992, the Chemical Weapons Convention, a treaty prohibiting the development, production, stockpiling, and use of chemical weapons and mandating their destruction, was ratified, and came into force in 1997 (Organization for the Prohibition of Chemical Weapons, 2019). However, in the interim, the Japanese doomsday cult group Aum Shinrikyo deployed GB in the Japanese city of Matsumoto in 1994, and then, more infamously, in the Tokyo subway system in 1995. In both these attacks, many defenseless people were poisoned, some fatally (Seto et al., 2000). The Tokyo subway sarin gas attack and the more recent US postal anthrax letter attacks in 2001 (Inglesby et al., 2002) presented a renewed threat of chemical and biological terrorism globally. In the Syrian war, many citizens were killed by GB and HD (John et al., 2018; United Nations, 2013). In 2017, Kim Jong Nam was assassinated using VX at Kuala Lumpur International Airport in Malaysia (Radio Free Asia, 2017). In 2018, a remerging nerve agent, Novichok, was used in Great Britain for assassination (Franca et al., 2019). To realize a safe and secure society, it is highly recommended that authorities at national levels establish a more strengthened crisis management system for civil defense (Seto, 2006a).

In addition to the known attacks, various CWA stockpiles have been discovered in former Japanese military force facilities during land excavations (Ohashi, 2004). Injuries due to direct contact with CWAs that leaked from containers in Samukawa, Japan, have been reported (Hanaoka, 2004), and some complaints were received in Kamisu, Japan, concerning damage to human health (neurological disorders) caused by drinking water likely contaminated with degradation products from arsenic vomiting agents (Ishii et al., 2004).

In the crisis management of chemical warfare terrorism cases (Society for Countermeasures Against Chemical, Biological, Radiological, Nuclear and Explosive Terrorism, 2008) and chemical weapon disposal, CWAs were monitored in public places, security checks at territorial borders, airports, large event venues, executive facilities, and demilitarization spots housing chemical weapons for protection against terrorism and workers' health. With regard to consequence management, on-site detection is initially performed by first responders for personal protection; on-site samples are then transported to laboratories for analysis for investigation and for identification of emergency lifesaving measures. In incident management, laboratory analysis is performed to provide evidence for courts in order to prosecute offenders and prevent future crimes (Fig. 57.1). Among these detection schemes, rapid on-site detection is the most important for the minimization of disasters in order to eliminate the prolonged times required for transporting on-site sample specimens to a laboratory for analysis (Smith, 2002; Harris, 2002; Fittch et al., 2003). Various types of measuring technologies have been used for on-site detection and laboratory identification of CWAs and are discussed here.

57.2 Properties of chemical warfare agents

CWAs are low-molecular-weight synthetic compounds that are fast-acting and sometimes lethal, even at low

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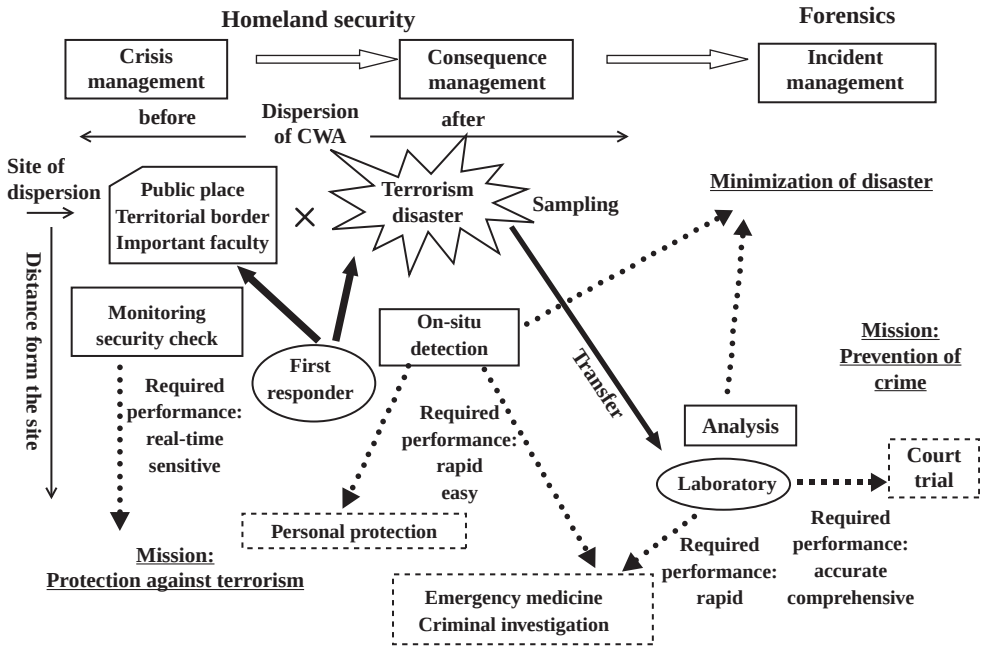

FIGURE 57.1 Detection and identification in chemical terrorism countermeasures and chemical weapon disposal.

levels (Somani, 1992; Stewart et al., 1992; Marrs et al., 1996; Ellison, 2000); in terms of physical properties, CWAs can be classified into gaseous blood agents, gaseous choking agents, volatile nerve agents, volatile blister agents, nonvolatile vomit agents, and nonvolatile lachrymators (tear gases) (Fig. 57.2). The physical and toxicological properties of CWAs vary in many ways; namely in molecular weight, melting point, boiling point (vapor pressure), vapor density, durability in air, lethal concentration, incapacitating concentration, smell, water solubility, stability in water, effect on skin, and antidote prospects. In particular, except for slow-acting agents that manifest toxicity after several hours, such as phosgene (CG) and HD, CWAs are fast-acting. Deadly poisonous organophosphate nerve agents and toxic blister agents are registered as scheduled compounds in the CWC. Although CWAs show toxicity by skin contact and intake, inhalation toxicity against CWA vapor is mainly considered with respect to chemical warfare/terrorism cases and chemical weapon disposal, and the development of analytical technologies is conducted with the aim of CWA detection in the vapor phase. The parameter used in the description of CWA toxicity is the lethal dose value (LCt_{50}, $mg \cdot min/m^3$); this value indicates the vapor concentration leading to a 50% incidence of death in human beings with 1 min of inhalation.

57.3 Concept of on-site detection

In chemical warfare terrorism countermeasures, we must consider the dispersion of various types of CWAs, including toxic industrial chemicals (TICs), except for overt cases such as previous notices of criminal acts. If clear symptoms of CWA-exposed casualties are manifest, it is possible to narrow down the types of CWAs detected. On the other hand, for chemical weapon disposal, the possible types of dispersed CWAs are obvious. In the case of exposure to CWA vapor and subsequent intoxication, monitoring technology is very important for exposure minimization and effective medical treatment. However, the requirements for the optimal performances of on-site detection technologies are not clear, with most protocols depending only on previous experience. Namely, the on-site monitoring of CWAs is quite different and an unknown field compared to other monitoring situations; therefore, not only researchers, but also first responders, have little idea of how to detect CWAs on site. Some of the required criteria for successful on-site detection are as follows: knowledge of the possible types of CWAs and their limits of alarm (LOAs), response time, time required to start the detector, recovery time after the previous alarm, accuracy (affected by interference and false positives), state of alarm, operational performance (i.e., the required technology and training), maintenance, and cost of introduction and maintenance. In addition, operational conditions, durability, and portability of necessary machinery should be considered.

For considering toxicity manifestation time and vapor dispersion situations in cases of terrorism, it is necessary to initiate the alarm for a vapor concentration of less than the vapor concentration of 1/100 of LCt_{50} within 1 min. In the case of GB, the required detection sensitivity is

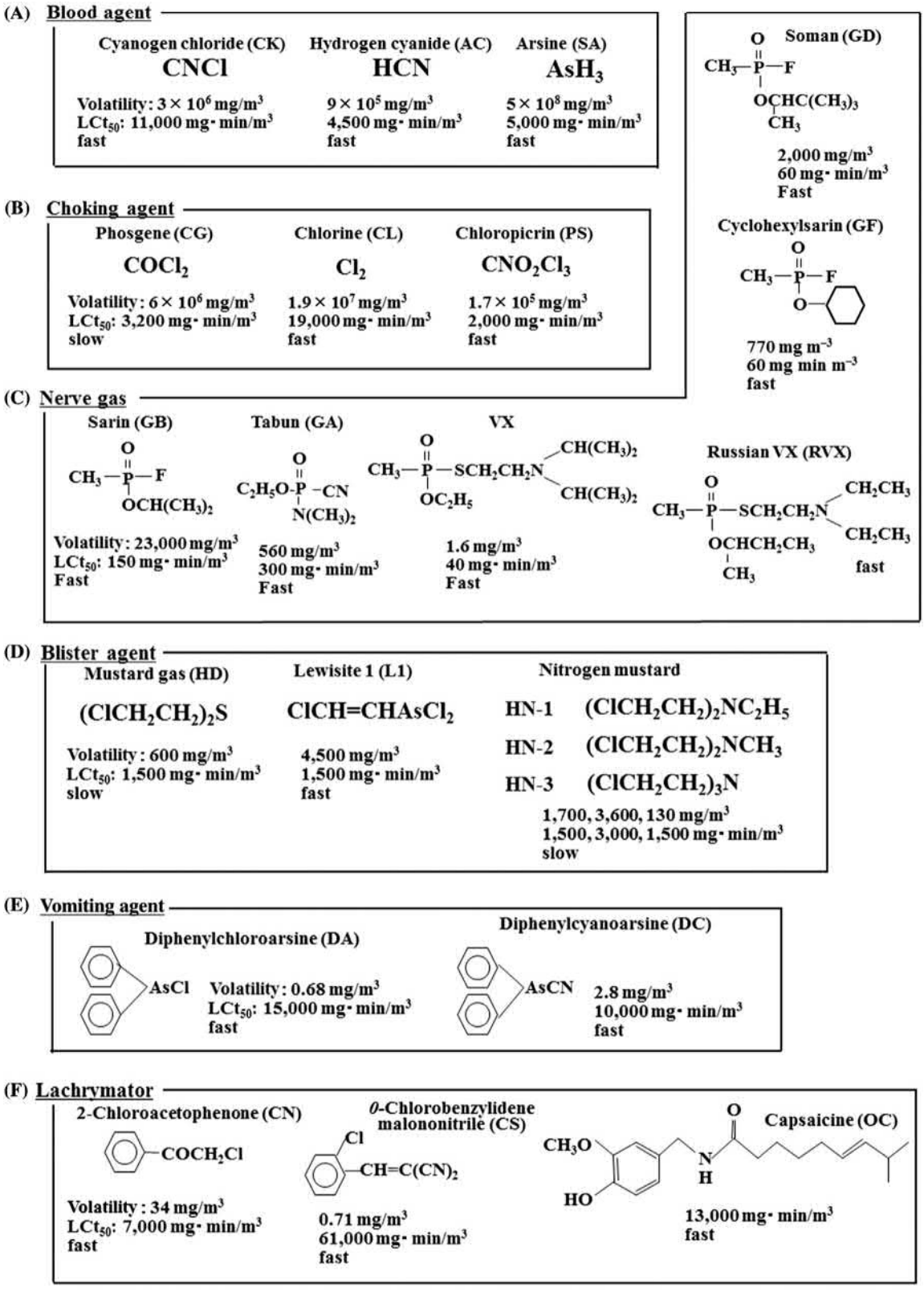

FIGURE 57.2 Chemical structures, volatility (at 25°C), and inhalation toxicity data of chemical warfare agents.

0.15 mg/m^3; at this level, humans show no signs of intoxication, nor is there a detectable odor. In chemical weapon disposal situations, because workers are present at the sites for a prolonged time, the time-weighted average (TWA) values, which are approximately 1/100,000 of LCt_{50}, are adopted as the monitoring target for allowed operational conditions. An alarm time of less than several minutes is desired. There is a tradeoff relationship between LOA, alarm time, detection accuracy, and operation; furthermore, for detection equipment employing one detection mechanism, raising the LOA prolongs the alarm time and increases the frequency of false positives.

In previous instances for which on-site detection technology had not yet been well developed, field damage and injuries to victims were traditionally observed and understood by using our senses; CWAs were identified through laboratory analysis and diagnostics after transferring the on-site samples and victims' samples to specific laboratories and emergency medical hospitals. To minimize the damage caused by terrorist activity, it is vital to obtain as much information as possible about the types and concentrations of the dispersed CWAs, which is now possible because of the technological development and large numbers of laboratory analytical technologies. Fig. 57.3 shows the concepts in the determination of CWAs. Remote detection (stand-off detection) is the method to detect CWAs away from the dispersed site using spectroscopic technologies, and can be divided into the following two types: (1) passive detection, which refers to the detection of light emitted from the target and is mostly limited to infrared light; and (2) active detection, which detects the secondary light (fluorescent or absorbed light—i.e., ultraviolet, visible, or infrared) emitted from the target after the sample is irradiated with primary light using equipment brought to the site, with lasers usually employed as the irradiating light source.

Because the vapor concentrations of CWAs used for terrorism are low due to their high toxicities, and terrorism is predicted to occur at civilian locations where various types of interfering compounds exist, it is often difficult to fulfill on-site detection requirements. "Suction detection" is the method to detect CWAs by directly measuring CWAs through suction of vapors in the field, and is further divided into the following two types: (1) continuous monitoring, which involves continuous drawing of on-site air samples and detection using fixed or movable equipment; and (2) point detection, which is used to detect CWA vapor by first responders by moving portable devices into the field. In terms of CWA detection, *real-time detection* refers to the alarm signal given under the continuous drawing of air samples, while in collection detection mode, the alarm is given by detection with cycling measurements after air samples have been collected. *Collection off-site analysis* refers to CWA detection in the laboratory using collected samples transferred from the field. For solid or liquid samples and dispersed surfaces, CWAs are detected by direct contact with the detector; alternatively, they are detected by measuring the sampled or stripped specimens by inserting them into the detectors. Finally, large trailers containing mobile chemical laboratories equipped with analytical machines are used for detection via on-site analysis.

The assessment of dangerous terrorism and chemical weapon disposal situations by detecting fast-acting CWAs quickly at low-concentration levels must be rapid (Sidell et al., 2003). For people escaping from or arriving at the dangerous sites, injuries and casualties must be taken care of with proper emergency treatment (such as securing aeration and administering antidotes). The roles of the first responders are to install protective masks and gear and to perform zoning of the dangerous sites. Field-deployability, rapid alarm capability, and ease of automated operation are most important for on-site detection, with the low frequency of false positives being a secondary requirement; however, if the frequency of false-positive alarms is high during a response, it will disturb the proper and timely activities of responders at the terrorism-affected or chemical weapon disposal sites. Various types of on-site CWA detection devices have been used by military organizations worldwide (Fittch et al., 2003), and some have been introduced for civil defense organizations such as mobile police teams, fire-defense teams, and coastguards for the purposes of counterterrorism.

CWAs can be measured by detecting the chemical constituent; the technologies for chemical detection can

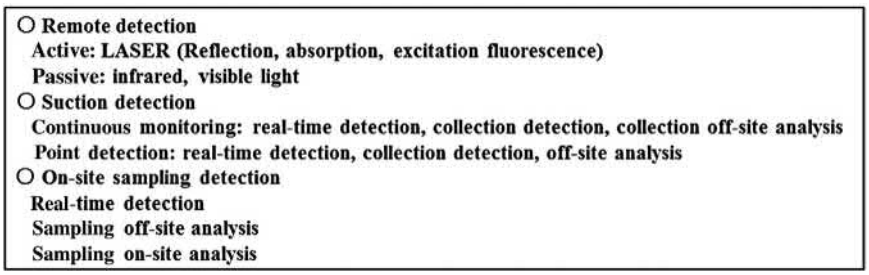

FIGURE 57.3 Concept of on-site detection for chemical warfare agents.

adopt different mechanisms depending on the target substances (Paddle, 1996). The discrimination level varies between the screening and identification methods. At present, most field equipment utilizes detection paper, gas detection tubes, ion mobility spectrometry (IMS), flame photometric detection (FPD), photoionization detection (PID), surface acoustic wave (SAW) detection, vibrational spectroscopy including Fourier transform/infrared spectrometry (FT/IR) and Raman spectroscopy, or combined GC-MS technologies. Furthermore, chemical sensors, biosensors, and micro total analysis systems (μTAS) have been extensively developed. Except for highly discriminating technologies such as chromatographic or spectrometric measurements, many detectors signal an alarm only for restricted chemical species. The representative species are nerve agents, blister agents, blood agents, including gaseous choking agents, and TICs. Vomit agents and lachrymators are not considered because of the mechanical impossibility for detecting such nonvolatile agents and their low toxicity. Nerve agents are the most preferred target, followed by blister and blood agents. So far, CWA detection technology has been developed and utilized for deployment during military missions; thus, it is not guaranteed that such on-site equipment would work properly for real CWAs in civil defense. Considering the present situation of developed terrorism countermeasures and on-site needs, greater emphasis should be placed on the scientific development of on-site detection technology. Several research groups are engaged in the evaluation of commercially available detection equipment and are presently developing new detection technologies. Our laboratory has evaluated some commercially available detection equipment using authentic CWAs. In the following section, the evaluation results obtained in the author's laboratory are discussed (Seto, 2006a, 2006b, 2014, 2015; Seto et al., 2005, 2007).

57.4 The present situation of detection technology

With regard to remote detection technology, the active method of measuring the reflected light from laser irradiation provides higher sensitivity than the passive method; however, the machinery for this technique is too large (on the ton scale). As for passive methods, spectroscopic measurements of CWA emission (specifically infrared light and the corresponding devices) were utilized in military applications. These machines can instantaneously and continuously detect the CWA vapor; however, because of background interference, practical utilization by civilian means remain a far-off prospect. Recently, remote-sensing technology using passive infrared light has been advanced (System Assessment and Validation for Emergency Responders Program, 2016), and now hyperspectral imaging machines are available (Bruker Corporation, 2019a,b). Furthermore, in addition to ultraviolet, visible, and infrared light, a wide range of other light wavelengths such as milliwave and terahertz light can be detected, in addition to laser excitation and resonance Raman scattering.

57.4.1 Classical manual method

The classical manual detection tools still in use are suction detection, detection paper, and gas detection tubes, which can detect the presence of CWAs by visualizing the color changes manifested by the reaction between the reagents and CWAs. Detection paper shows an instant color change in the presence of liquid forms of CWAs with a sensitivity of several $\mu g/cm^2$ (Toyobo, Japan; M8 and M9 paper, US military). Two types of pigments and pH indicators are impregnated in the cellulose paper; the paper turns brown or orange on the addition of a droplet of GB, soman (GD), or tabun (GA) [G agents in the North Atlantic Treaty Organization (NATO) code]; turns red when exposed to a droplet of HD or lewisite 1 (L1) (H agents); and turns to black or deep green with VX (a V agent). This coloring reaction is based on the solubility of the agents in organic solvent; thus, almost all organic solvents except water would show a false positive. Dimethyl methylphosphonate, acetone, toluene, and ethyl acetate show a false positive for G agents; 2-mercaptoethanol, carbon tetrachloride, and aniline show a false positive for H agents; and diethylamine shows a false positive for V agents. Because of the high probability of false positives, detection paper seems impractical for civilian defense (Seto et al., 2005).

Gas detection tubes show a color change with vapor CWAs with a sensitivity close to mg/m^3 concentration levels. The specific reagents are impregnated into a silica-gel support in a glass tube. When needed, both sides of the tube are opened by a cutter, the appropriate air sample volume is drawn, and the extent or length of coloring is checked by the naked eye. Commercial industrial-use gas detection tubes (Komyo Kika; Gastec, Japan) are available for the detection of blood and choking agents. However, for special agents such as nerve agents, the Dräger Safety gas detection tube (in Germany) is adequate (Takayama et al., 2007).

For nerve agents, the phosphoric acid ester tube shows a red color, which is based on a sequence of complicated procedures involving butyrylcholinesterase (BuChE) inhibition, substrate butyrylcholine hydrolysis, and pH indication. Positive results are shown for cholinesterase inhibitors. For HD, the thioether tube exhibits a yellow color, which is based on the reaction with silver chloride

and chloramines. HD stimulants show false positives. For L1, the arsine and organic arsenicals tube shows a black color; a sequential procedure involving two types of chemical reactions allows the separate detection of both inorganic and organic arsenicals and, based on the reduction by zinc and hydrochloride and subsequent complex formation with gold and mercury, yields a gold-colored colloid. For nitrogen mustard 1 (HN 1), nitrogen mustard 2 (HN 2), and nitrogen mustard 3 (HN 3), the organic alkaline nitrogen compounds tube shows an orange-red color, which is based on the Dragendorf reaction. For hydrogen cyanide (AC), the hydrogen cyanide tube shows a pink color, which is based on oxidation by mercury chloride and subsequent pH indication. For cyanogen chloride (CK), the cyanogen chloride tube also shows a pink color, which is based on the reaction with pyridine and barbituric acid. For CG, the phosgene tube shows a blue-green color, which is based on the chemical reaction with *p*-dimethylbenzaldehyde/*N,N*-dimethylaniline. For chlorine (CL), the chlorine tube shows an orange color, which is based on a chemical reaction with *o*-tolidine. The LOA (i.e., the minimum concentration giving three positive results within three trials), response time, and interference of the stimulants and solvents are shown in Table 57.1. The operation involves a complicated procedure of breaking the inner liquid tube and subsequent incubation in a number of detection tubes. However, the procedure is quite tedious when employing protective gear and gloves, requiring several minutes from the start of the operation until tube coloration is observed. The gas detection tube is recommended as a supplemental means for ascertainment of CWA species after IMS screening.

TABLE 57.1 Detection performance of Dräger gas detection tubes.

Gas detection tube	Agent	Limit of alarm (mg/m^3)	Response time (min)	Remark
Phosphoric acid ester	Sarin	0.002	5–6	
	Soman	0.02	5–6	
	Tabun	0.5	5–6	
	VX	2	5–6	
	Dichlorvos			False positive at 1 mg/m^3
	Methomyl			False positive at 50 mg/m^3
	Dimethylmethyl-phosphonate			Negative at 2300 mg/m^3
Thioether	Mustard gas	2	2	
	2-Chloroethylethyl sulfide			False positive at 2.4 mg/m^3
	1,4-Thioxane			False positive at 2.4 mg/m^3
	Diethylether			Negative at 20000 mg/m^3
Organic arsenic compound and arsine	Lewisite 1	4	2	
	Diphenylchloro-arsine			Negative at 100 mg/m^3
Organic alkaline nitrogen compounds	Nitrogen mustard 1	10	1	
	Nitrogen mustard 2	10	1	
	Nitrogen mustard 3	10	1	
Hydrogen cyanide	Hydrogen cyanide	0.3	1	
	Cyanogen chloride			Negative at 100 mg/m^3
Cyanogen chloride	Cyanogen chloride			
	Hydrogen cyanide			Negative at 100 mg/m^3
Phosgene	Phosgene	1	1	
Chlorine	Chlorine	0.8	1	

57.4.2 Photometric method

The photometric type of detection is based on the photometric or electronic response manifested by the physical-chemical reaction of CWAs. The FPD instrument detects CWAs by measuring the specific phosphorus or sulfur emission line produced via combustion with hydrogen gas. AP2C (PROENGIN, 2019) is a handy portable automated FPD manufactured by Proengin (based in Saint Cyr l'Ecole, France). This detector responds rapidly to both vapor and liquid forms of phosphorus containing CWAs in the GV mode and of sulfur-containing CWAs in the H mode. Approximate concentration levels of 0.1 and 1 mg/m^3 for nerve agents and HD are detected in the GV and H modes, respectively (Seto et al., 2005, 2007). The stimulants containing phosphorus or sulfur show false positivity, and CWAs containing neither phosphorus nor sulfur are not detected. A flame ionization detection (FID) instrument such as MicroFID II (INFICON, 2019a) detects nonspecific combustible CWAs by measuring ion production manifested by combustion with hydrogen; the LOA of this method is at sub-mg/m^3 levels of vapor concentrations. The PID instrument detects numerous types of CWAs in a nonspecific fashion, which is based on the measurement of the produced ion current of charged gas ions by irradiation with ultraviolet light. RAE Systems (based in San Jose, California) manufactures the ppbRAE 3000 +, which has an LOA on the level of several tens of mg/m^3 (Honeywell International Inc., 2019; Seto et al., 2007).

Chemical sensor technology adopting acoustic waves (Harris, 2002) is also available for CWA on-site detection. The CWAs are adsorbed reversibly onto the arrayed cells of the specific liquid phase polymer, and the acoustic wave numbers change according to the mass increase due to CWA adsorption. CWAs can be detected and discriminated by analyzing the respective wave number changes (Harris, 2003; Grate, 2000); discriminating power is increased by increasing the number of polymer cells. Portable detectors adopting arrayed SAW devices are commercially available. ChemSentry, manufactured by BAE Systems (USA), adopts 10 different polymer cells and identifies CWAs as "NERV" for nerve agents, "BL" for blister agents, and "BLOOD" for blood and choking agents. The LOA is rather high, the response and recovery time is long, and the number of false positives is high (Matsushita et al., 2005). This chemo-adsorptive sensor technology provides the possibility of increased discrimination by adopting arrayed cells, but because of the strong adsorption of CWAs, disturbs quick and reversible binding on the polymer arrays.

57.4.3 Ion mobility spectrometry method

The IMS instrument is most frequently used for military and civil defense missions to detect not only CWAs (Makinen et al., 2010), but also explosives and illicit drugs (Eiceman and Stone, 2004; Eiceman and Karpas, 2013). The drawn air sample is ionized commonly by a $\beta-$ emitter or through corona discharge under atmospheric pressure, and the ionized water-cluster molecules (reactant ion peak, RIP) react with CWA molecules in the reaction region. The generated cluster ions (positive or negative) periodically traverse through the drift region and are detected on the collector. The ion mobility depends on their charge and molecular mass over cycles of several milliseconds (Fig. 57.4). CWAs are recognized according to ion mobility of the resulting peak or peaks, and semiquantified according to the ion peak height (St Louis et al., 1990). In the positive ion mode, a protonated water cluster combines with the targets; in the negative ion mode, a water and carbon dioxide cluster attached to oxygen combines with the targets, and ammonia or organic solvents are introduced as dopants (Puton et al., 2008) into the drift region, which raises the sensitivity and discriminating power. Easily ionizable nerve agents show high sensitivity, while weakly ionizable blister agents do not. Low-molecular-weight blood and choking agents do not produce characteristic cluster ions; therefore, detection sensitivity is low. Because of atmospheric pressure ionization, the devices are manufactured to be rather compact, and their response times are fast. However, the resolution of the produced target ions is low compared to MS, leading to frequent false positives by many types of organic solvents. To raise the sensitivity and accuracy, the ionization mechanism has been improved using electrospray, laser desorption, matrix-assisted laser desorption, and the development of new hardware (Kolakowski and Mester, 2007). MS machines are combined with an IMS instrument for molecular assignment of the produced ions (Kanu et al., 2008). Field-portable instruments based on field asymmetric IMS (FAIMS) and differential mobility spectrometry (Guevremont, 2004) are now commercially available (Chemring Group, 2019). A typical FAIMS result for HD measured by an Owlstone FAIMS machine (Owlstone Inc., 2019) is shown in Fig. 57.5.

An aspiration-type device is based on discrimination by the pattern recognition of several IMS cells of different ion mobilities and polarities (Utriainen et al., 2003; Zimmermann et al., 2007). Environics Oy (based in Mikkeli, Finland) manufactures M90 (type D1-C), an aspiration-type 160 μCi ^{241}Am-bearing IMS, adopting six IMS cells and one semiconductor cell, which provides vapor detection of GB, GD, GA, and GF (LOA: sub-mg/m^3) as a NERVE mode alarm with a false-positive alarm against vapors of phosphorus stimulants such as dimethyl methylphosphonate. HD, L1, and HN 3 vapors (LOA: several mg/m^3) cause a BLISTER mode alarm; however, HD stimulants such as 2-chloroethyl ethyl sulfide vapor also cause a false-positive alarm. AC or CK gas shows a

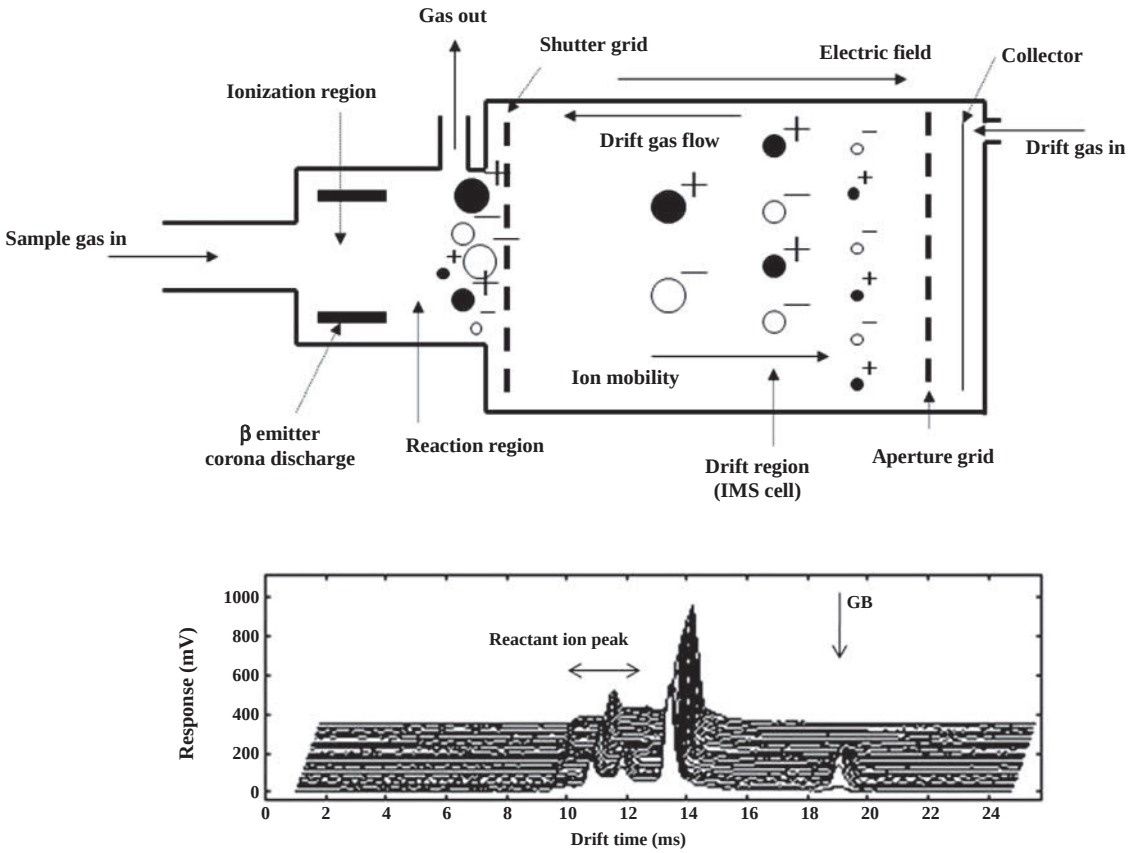

FIGURE 57.4 Ion mobility spectrometry. Detection mechanism and an exemplified spectrum. SABRE4000 (Smiths Detections Ltd., ionization ^{63}N) detected GB.

false BLISTER alarm. CG and CL cause a BLOOD mode alarm. The LOA is rather low for volatile CWAs and high for gaseous CWAs, the response and recovery time is short, and the frequency of false positives is moderate (Kishi et al., 2010). An aspiration-type IMS, ChemPro100i, from the same Finnish company, adopts 16 IMS cells and one semiconductor cell (Zimmermann et al., 2008), providing similar recognition (Environics Oy, 2019).

LCD-3.2E, a corona discharge-type short drift tube IMS instrument, developed by Smiths Detection (based in London), is a useful detector showing two types of alarm: "G" for nerve agents, and "H" for other CWAs (Sekioka et al., 2007). The advanced version (LCD 3.3) provides an agent-name alarm (Smiths Detection, 2019a). This machine adopts an ammonia dopant, raising the sensitivity and discriminating power. Maintenance requires that the sieve pack be frequently exchanged for proper operation. Tables 57.2 and 57.3 show the LOA, response and recovery times, and interference toward CWAs (Satoh et al., 2015). SABRE 4000, also manufactured by Smiths Detection, is a long-drift tube (high-resolution) IMS providing an agent-name alarm. As shown in Tables 57.4 and 57.5, the LOA is rather low, the response and recovery time is short, and the frequency of false positives is low (Yamaguchi et al., 2010). AIRSENSE (based in Schwerin, Germany) manufactures GDA2, a multisensor system consisting of an IMS, a semiconductor, a metal oxide, and a PID, enabling identification and quantification of not only CWAs, but also TICs (AIRSENSE Analytics, 2019).

Discriminative detection of nerve agents and blister agents is possible because the positive marker ions formed from such agents are well separated. However, detection of gaseous CWAs, blood agents, and choking agents, is comparably difficult. The negative maker ions formed from such agents cannot be separated from the RIP under the ionization of β-emitter or corona discharge, because the RIP in the negative ion mode is composed of various ions with a relatively wide peak shape. Atmospheric electron emission is another ionization where only superoxide anion is produced as the negative RIP, and specific marker ions are formed from gaseous CWAs and well separated from the RIP (O_3^-), enabling discriminative detection (Seto et al., 2019). Schematics of the atmospheric electron emission device and the typical spectra are shown in Fig. 57.6.

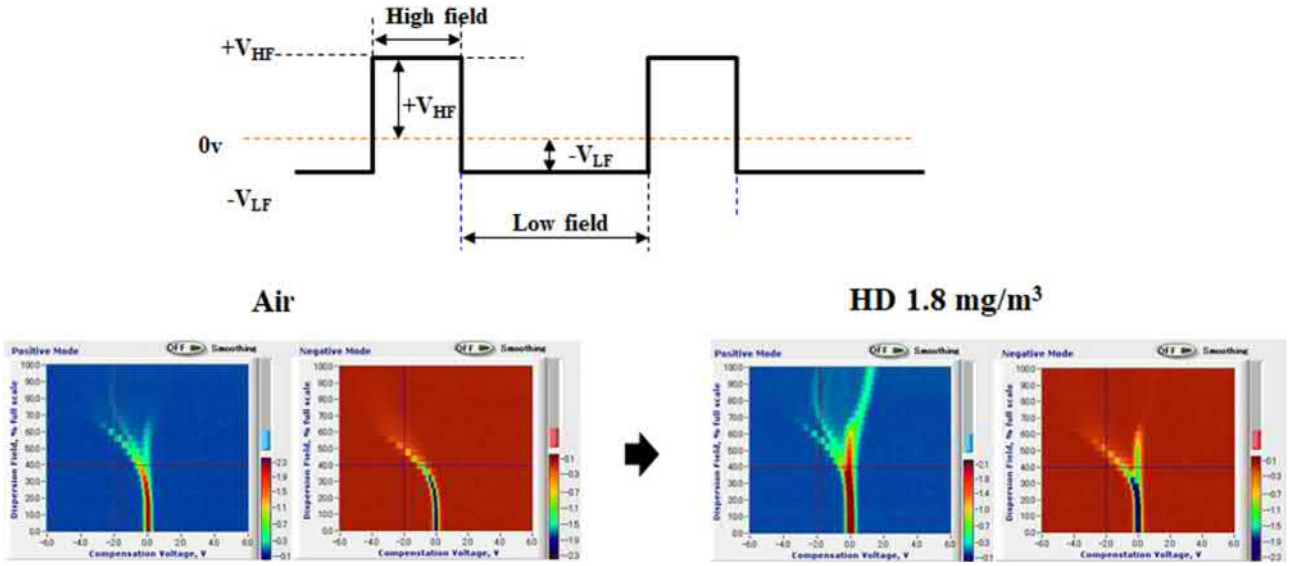

FIGURE 57.5 Detection of sulfur mustard by field asymmetric ion mobility spectrometry. Upper chart shows the electric field control. Lower charts shows the dispersion plots. (Left) Background air sample flow (left: positive ion mode; right: negative ion mode); (right) sulfur mustard (1.8 mg/m^3 and *n*-hexane 1320 mg/m^3 in air).

TABLE 57.2 Detection performance of corona discharge-type short drift tube IMS detector LCD-3.3.

Agent	Alarm	Limit of alarm (mg/m^3)	Response time (s)	Drift time (s)	Reduced ion mobility (k_o)
Reactant ion peak				+4.8, −4.9	+2.28, −2.16
Sarin	GB	0.1	5–12	+7.1, +9.0	+1.56, +1.25
Soman	GD/GF	0.3	3–28	+8.1, +10.8	+1.35, +1.04
Tabun	GA, HD	0.2	13–30	+7.5, +9.8, −6.9	+1.44, +1.10, −1.46
Cyclohexylsarin	GD/GF	1	14–32	+8.0, +10.5	+1.36, +1.04
VX	VX, L	10	8–27	+6.8, +8.3, −6.3	+1.62, +1.31, −1.58
RVX	VXR, VX	3	7–25	+7.7, +8.6	+1.42, +1.28
Mustard gas	HD	0.4	4–13	−6.8	−1.47
Lewisite 1	L	3.8	5	2	
Nitrogen mustard 1	(−)[a]			+6.7	+1.62
Nitrogen mustard 2	VX, HD	200	5–10	+6.6, −6.9	+1.64, −1.48
Nitrogen mustard 3	HN, GB	1	3–13	+5.8, +7.0, −7.5	+1.86, +1.56, −1.34
Hydrogen cyanide	AC, CK	6	3–9	−4.3	−2.33
Cyanogen chloride	CK	100	20–27	−4.5	−2.22
Phosgene	AC	20	5–12	−4.4	−2.29
Chlorine	CK	5	2–17	−4.6	−2.18
Chloropicrin	AC	200	9–28	−4.3	−2.32
2-Chloroacetopheone	GA	3	8–23	+6.5, +7.6	+1.67, +1.35
o-Chlorobenzylidenemalononitrile	(−)			−7.3	−1.38
Methylisothiocyanate	(−)			6.0	−1.61

[a]*No alarm.*

TABLE 57.3 Response of LCD 3.3 against organic solvents.

Alarm	Compound
GB	2-Mercaptoethanol, hydrochloric acid, triethylamine, dimethylfolmamide, *N*-methylamiline, methylisobutylketone
GD/GF	Diisopropylfluorophosphate, *n*-nonane
GA	Triethylphosphate, *n*-nonane, tetrahydrofuran, propionic acid, *N*-methylaniline, *N*-ethylaniline
RVX	Dimethylmethylphosphonate, trimethylphosphate, *N*,*N*-diethylaniline, methylisobutylketone
HN	Methylisobutylketone
CK	Chloroform
HD	2-Mercaptoethanol, 1,4-thioxane, 2-mercaptopropanol, tetrahydrofuran, acetic acid, propionic acid, methylisobutylketone
Negative	32 solvents

TABLE 57.4 Detection performance of ^{63}Ni-ionization-type long drift tube IMS detector SABRE 4000.

Agent	Polarity	Alarm	Limit of alarm (mg/m^3)	Response time (s)	Remarks
Sarin	+	GB	0.008	4–5	
Soman	+	GD/GF	0.02	2–5	
Cyclohexylsarin	+	GF	0.45	2	
Tabun	+	GA	0.08	4–6	
Dimethylmethyl-phosphonate		(–)[a]	2300		Negative
Trimethylphosphate		(–)	2400		Negative
Triethylphospahte		(–)	2400		Negative
Dichlorvos	–	HD/Phos	14		False positive
Mustard gas	–	HD/Phos	0.48	3	
Lewisite 1	–	Acids	19	15	
Nitrogen mustard 1	+	VERIFIC	0.044	2	
	–	HD/Phos	0.44	8	
Nitrogen mustard 2	1.2	HD/Phos	1.2	2–4	
Nitrogen mustard 3	+	HN3	0.0048	6–9	
	–	HD/Phos	1.2		
2-Chloroethylethyl-sulfide	–	HD/Phos	1.1		False positive
1,4-Thioxane	–	HCN	110		False positive
		HD/Phos			
2-Mercaptoethanol	–	Acids	2200		
Hydrogen cyanide	–	HCN	0.2	2–4	
Cyanogen chloride	–	HCN	5	1–2	
Phosgene	–	HD/Phos	1.5	2–4	
Chlorine	–	(–)			Negative at 130 mg/m^3
Chloropicrin	–	HD/Phos	0.13	4–5	

[a] *No alarm.*

TABLE 57.5 Response of SABRE 4000 against organic solvents.

Alarm	Compound
HN3	Ethyl acetate
Acids	Diethylamine
Negative	26 solvents

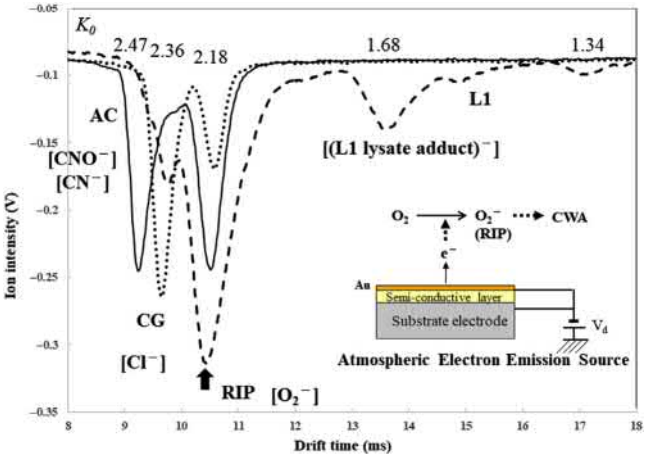

FIGURE 57.6 Negative ion mode ion mobility spectra of hydrogen cyanide (9 mg/m^3, solid line), phosgene (442 mg/m^3, dotted line) and lewisite 1 (50 mg/m^3, broken line) with atmospheric electron emission ionization. The charging voltage for electron emission, the drift length, and voltage of the IMS instrument were 20 V, 7 cm, and 1981 V, respectively. The K_0 values and the chemical structures for the separated ion peaks are shown.

57.4.4 Vibrational spectroscopy

The FT/IR instrument detects and identifies CWAs by noninvasively and instantly measuring the infrared spectrum of the air sample (Mukhopadhyay, 2004). Because the absorption coefficients of the IR band peaks are relatively low, the light path lengths should be long to raise the detection sensitivity. Considering the interference of water and carbon dioxide, their characteristic absorbance peaks in the low-wave number region (fingerprint region) are used as specific markers. Open path FT-IR has been developed to detect CWAs spread in the field by measuring IR spectra in the open space between the detector and the retroreflector at the distal end, achieving the path length over 100 m (Griffiths et al., 2009). Alternatively, the multiple reflection method has been used to lengthen the light path in the sample cell to achieve a path length of about 10 m, and the portable automated machines are now commercially available (Gasmet, 2019). MKS Instruments (based in Andover, Massachusetts) provides an automated FT-IR machine, AIRGARD (MKS Instruments, 2019), which adopts about 10 m multiple reflection path length gas cell (0.4 L), high flow rate (6–10 L/min) and rolling background algorism, enabling highly sensitive identification (LOD: around 0.1 mg/m^3) without background interference (Phillips and Tan, 2010). Fig. 57.7 shows the FT-IR operational flow and the detection result of GB. A sensitive method is also reported using an attenuated total reflectance device by adsorbing the target analytes from the vapor onto the solid-phase microextraction adsorbent (Bryant et al., 2007). Smiths Detection provides a portable FT-IR instrument, HazMatID (Smiths Detection, 2019b), which uses a diamond sensor and an extended onboard spectral library containing spectra for many organic compounds, including CWAs and suspected white powder materials.

A handful of Raman spectroscopy instruments are commercially available to match the identification of unknown liquid and solid samples in the forensic and homeland security regions (Izake, 2010), including First Defender (Thermo Scientific, 2019). Rigaku Corporation (based in Tokyo, Japan) provides a Raman spectrometer, ProgenyResQ CQL, which allows the quick identification of unknown solid and liquid chemicals using a vast sample library, including explosives, TICs, CWAs, white powders, and narcotics. This machine adopts a laser excitation of 1054 nm, preventing interference from fluorescent compounds (Rigaku Corporation, 2019). As shown in Fig. 57.8, 1054 nm excitation is superior to 785 nm excitation in identifying CWAs. In 785 nm excitation, Raman spectra are ill-shaped for HN3 and a mixture of L1 and gasoline (Kondo et al., 2018)

57.4.5 Gas chromatography

The GC instrument detects CWAs by measuring the peak response appearing on the GC column (Makas and Troshkov, 2004). CWAs are distinguished on the basis of retention time and their detectability by specific detection devices (Henry, 1997). Combined with an automated air collection–thermal desorption system, GC provides very sensitive detection (lower than μg/m^3) of nerve agents and blister agents over a 10-min. cycle. O. I. Corporation (based in Pelham, Alabama) manufactures the movable GC-based MINICAMS (O.I. Corporation/Xylem Inc., 2019). GC can be hyphenated with other detection systems, such as SAW (Williams and Pappas, 1999) and IMS (Buryakov, 2004; GAS, 2019), enabling it to be used to detect CWAs while having a compact body and field portability.

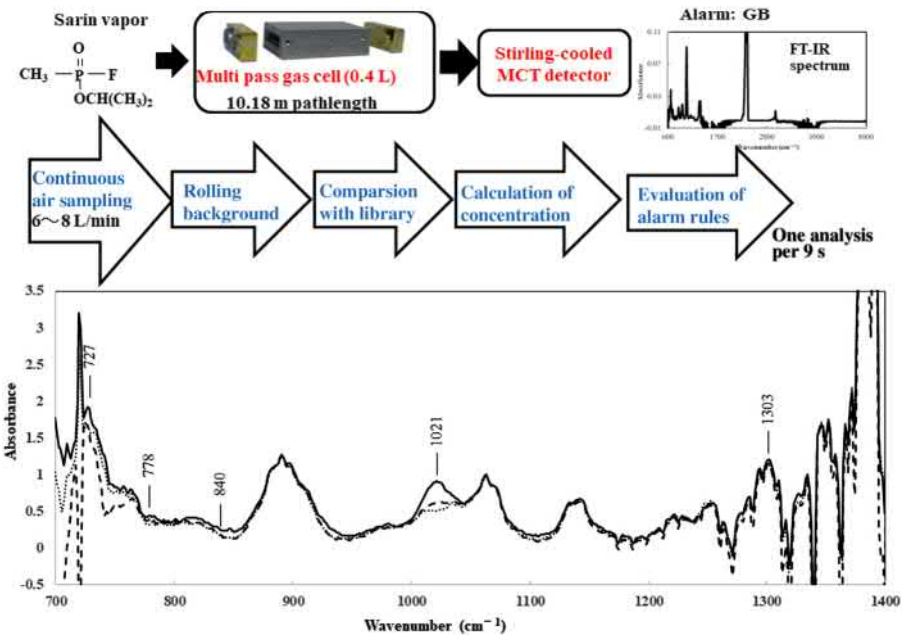

FIGURE 57.7 (A) Portable Fourier-transformed infrared spectroscopy instrument, AIRGARD; (B) operational flow; (C) fingerprint regions of infrared spectra of sarin in *n*-hexane 1320 mg/m^3 in air. Dotted line: sarin 0 mg/m^3; broken line: sarin 0.66 mg/m^3; solid line: sarin 2.2 mg/m^3.

57.4.6 Mass spectrometry

By miniaturizing the laboratory-type MS instrument and making it portable and resistant to mechanical shock, the machine can be used to detect CWAs in the field (Wise and Guerin, 1997; Smith et al., 2011a). MS provides high-resolution power, but to maintain a vacuum, the device must be large, complicating its operation. Electron ionization is usually used because of the benefit of abundant mass spectra data libraries availability (Virkki et al., 1995). On-site MS adopts a hydrophobic membrane inlet system to eliminate oxygen and nitrogen from the ionization region. The adsorbed CWAs are next introduced to the ionization region. The small molecules and strong adsorptive compounds cannot be detected by this ionization-type MS. Vapor forms of CWAs can be directly measured by atmospheric pressure chemical ionization (APCI) MS technologies such as direct analysis in real time (DART) (Nilles et al., 2009), selective ion flow MS (Francis et al., 2009), chemical ionization reaction time-of-flight MS (Cordell et al., 2007), and plasma desorption ionization MS (Iwai et al, 2015). The Riken group (based in Wako, Japan) and the author have developed an elemental analytical system using an electron cyclotron resonance ion source MS to detect CWAs (Urabe et al., 2014).

GC-MS technology can also be downsized for field use (Henry, 1997). Resistive column heating (low thermal mass) technology (Sloan et al., 2001) provides rapid on-site confirmation of a wide range of CWAs. Inficon (based in Bad Ragaz, Switzerland) manufactures Hapsite, a field-portable GC-MS instrument (INFICON Co., Ltd, 2019b). Vapor is withdrawn for 30 s into the Tenax preconcentration system and thermally desorbed into a nonpolar capillary column with an elevated temperature control; the separated components are finally analyzed using an electron ionization quadrupole mass spectrometer. GB, GD, GA, and HD are detected and identified within 12 min, with postulated LOD values of 0.2, 0.5, 8, and 0.3 μg/m^3 (Sekiguchi et al., 2006). The new type of the same instrument, Hapsite ER, adopting Tri-Bed TA microconcentration, provides excellent determination against CWAs (Fig. 57.9). Even gaseous CWAs can be detected by direct gas analysis by bypassing the microconcentration and GC separation (Nagashima et al., 2015). For nonvolatile nerve agents, VX and RVX, the VX-G conversion tube is used to convert VX and RVX to volatile ethylsarin and isobutylsarin by fluoridation reaction (Ohrui et al., 2017). PerkinElmer Inc. (based in Waltham, Massachusetts) produces a field-portable GC-toroidal ion-trap MS machine, Torion T-9 with a solid-phase microextraction sampling inlet for CWA and related compound determination in water samples (PerkinElmer Inc., 2019; Contreras et al., 2008; Smith et al., 2011b).

57.4.7 Other sensor technologies

As for sensor technologies, chemical sensors can be used for detecting CWAs. Electrochemical sensors composed of multiwalled carbon nanotubes on indium tin oxide surfaces in connection with ferrocene–amino acid conjugates have been developed for detecting nerve agent

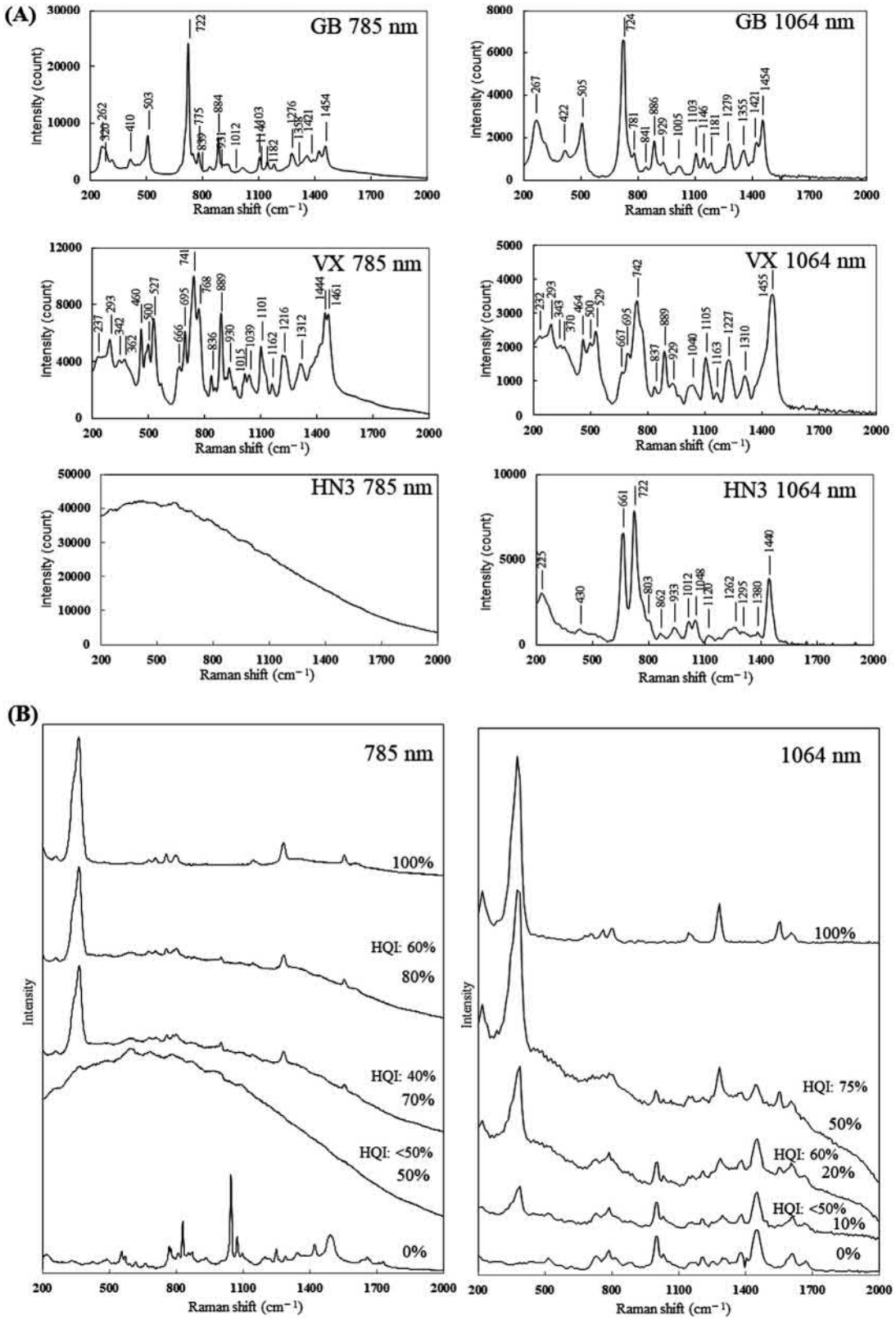

FIGURE 57.8 Raman spectra of chemical warfare agents. (Upper chart) Raman spectra of neat sarin, VX and nitrogen mustard 3 measured with the excitation of 785 and 1064 nm. (Lower charts) Raman spectra of the mixture solutions of lewisite 1 and gasoline measured with the excitation of 785 and 1064 nm.

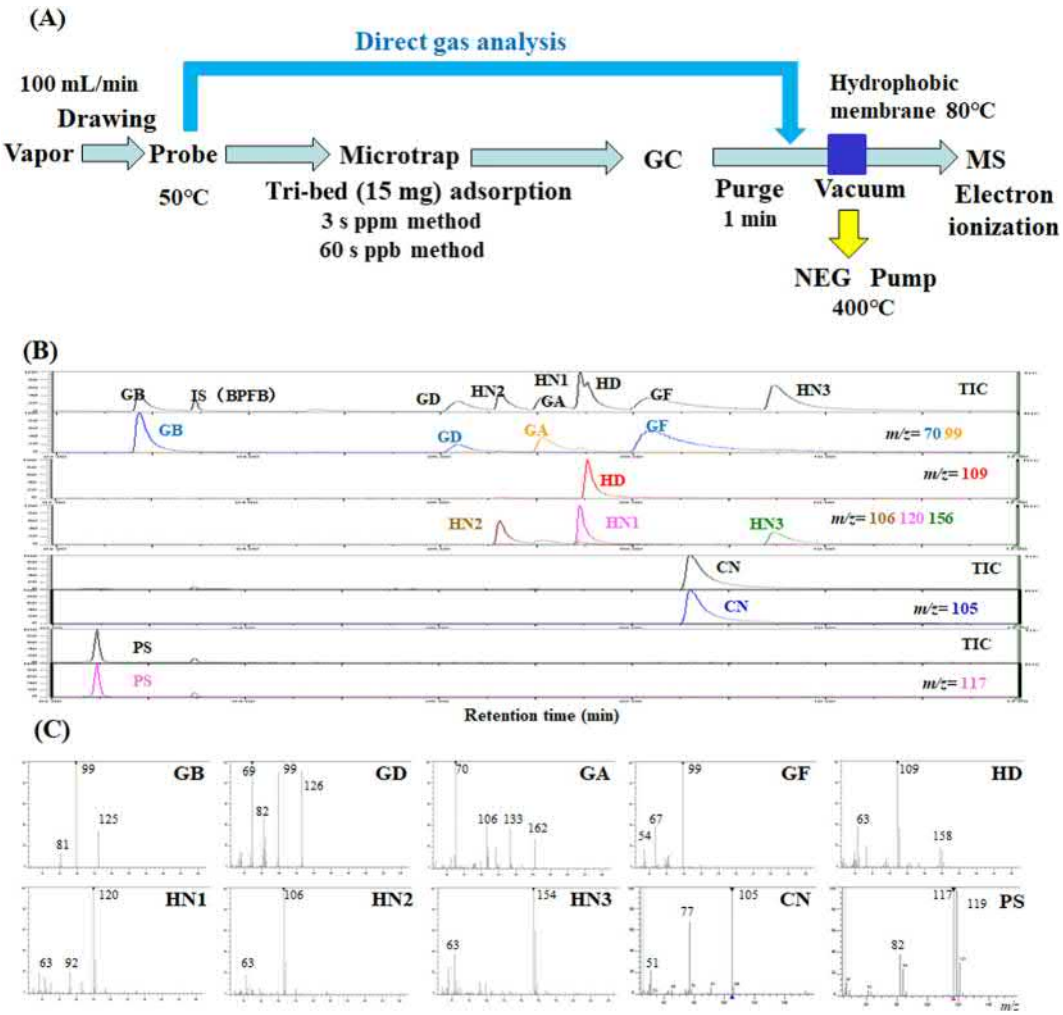

FIGURE 57.9 Portable gas chromatography-mass spectrometry instrument, Hapsite ER. (A) Operational flow; (B) total and extracted ion chromatograms; (C) electron ionization mass spectra. CWAs were measured using a HAPSITE ER. GB: 34 mg/m^3; GD: 10 mg/m^3; GA: 22 mg/m^3; GF: 14 mg/m^3; HD: 2.4 mg/m^3; HN1: 6.5 mg/m^3; HN2: 12 mg/m^3; HN3: 7.4 mg/m^3; CN: 2 mg/m^3; PS 3.7 mg/m^3; IS: internal standard (BPFB). Analytical conditions: microtrap desorption, Tri-Bed TA 15 mg, 5 s at 60°C, to 180°C; column, low thermal mass DB-1 0.25 mm × 15 m, 1 μm thickness), flow: nitrogen 2 mL/min; oven: 60°C (1 min), 6°C/min to 80°C, 12°C/min to 120°C, 26°C/min to 180°C (2 min); temperatures, membrane 80°C, valve oven 70°C, probe 60°C, NEG 400°C; MS, electron ionization, $m/z = 45-300$, 70 eV, 300 μA, 0.79 s/scan.

degradation products and related compounds in water (Khan et al., 2008). Electrochemical sensors developed by Riken Keiki Ltd. (based in Tokyo, Japan) provide sensitive and specific detection of gaseous CWAs (Riken Keiki Co., Ltd, 2019), and newly developed gold nanoparticle dispersed carbon fiber electrodes to selectively detect blistering agents in cooperation with Kumamoto University (in Kumamoto, Japan) and our group (Matsuura et al., 2010). The field-portable instrument (SC-90, 1.9 kg) was able to detect HD vapor with an LOA of 1.5 mg/m^3.

Biosensors utilizing CWA target enzymes have been developed over the past decades (Guerrieri et al., 2005; Zayats et al., 2003; Walker and Asher, 2005). Acetylcholinesterase (AChE) is fixed on the sensor chip for nerve agent detection; by reaction with nerve agents, substrate hydrolysis velocity is lowered because of enzyme inhibition (Anzai, 2015), and the resulting electric response from a reaction, such as the formation of hydrogen peroxide by choline oxidase, can be monitored (Palleschi et al., 1992). A biosensor device utilizing organophosphorus hydrolase (Russell et al., 1999; Karnati et al., 2007) and a chemical sensor utilizing nerve agent-reactive fluorescent reagent (Zhang and Swager, 2003) have also been developed. Sanders et al. developed a unique, remote, tissue-based biosensor for the field detection of GB, GD, and HD vapors by photosynthetic fluorescence induction measurements with target cyanobacteria (Sanders et al., 2001). However, these types of sensors are still not available for practical use. To detect AC, tyrosinase is fixed onto glassy

carbon; after increasing the concentration of AC on the colloidal mineral, its inhibition is monitored by electrochemically measuring polyphenol hydrolysis (Shan et al., 2004). Nerve agents and blister agents easily hydrolyze to form characteristic compounds, and the hydrolyzed CWAs can be analyzed in wet systems utilizing μTAS capillary electrophoresis (Wang, 2004). A field-portable multiple biosensing instrument has been developed for detecting complex hazardous material of nerve agents, biological toxin ricin, and *Bacillus anthracis* bacteria (Saito et al., 2018), where GB and VX in the aerosol are collected with mist by a cyclone unit, microfluidically delivered to the reaction cell mixed with the recombinant human AChE and acetylthiocholine (substrate), and the remaining substrate is measured electrochemically on screen-printed carbon electrode. Nine ng/m^3 GB could be detected.

57.5 Comparison of existing on-site detection technologies

In terms of required detection performance criteria for use by first responders, the detection sensitivity, detection accuracy, response time, recovery time, and operation are selected; among the detection equipment examined, these items are compared in Table 57.6. The numerical data are derived from our experiments. The evaluations are grouped into three categories (OK, Δ, X). A perfect device cannot be assigned that meets all the CWA detection requirements. Low sensitivity, false alarms, and strong adsorption of CWAs on devices are particularly serious problems. The gas detection tube system, which permits the detection of a wide range of CWA vapors, suffers from tedious operation and a slow response. The IMS-based detectors, which permit rapid and sensitive detection of nerve and blistering agents, show low sensitivity to gaseous agents and false positives in response to some compounds. The FPD-based detector, which permits rapid and sensitive detection of nerve agents and HD, gives false negatives when exposed to nonphosphorus and sulfur CWAs. The PID-based detector shows nonspecific and low detection sensitivity. The SAW-based detector does not permit sensitive detection of CWAs. The FT-IR instrument, which permits noninvasive and constant detection, shows moderate sensitivity. The GC-based detector with a concentration system, which permits sensitive detection of nerve agents and blister agents, suffers from tedious operation and a slow response. The GC-MS-based detector, which permits sensitive identification of almost all kinds of CWAS, suffers from complicated operation and slow response. Nonvolatile vomit agents and lachrymators cannot be detected using any of these equipments.

57.6 Development of new on-site detection technologies

Fig. 57.10 shows a performance map of on-site CWA detection equipment distributed by physical properties of the agents. CWAs are represented in terms of volatility and molecular weight, and the target agent territories and drawbacks of the detection equipment can be noted. This figure appears to show that the detection of gaseous agents and nonvolatile CWAs should be improved. For achieving a more sensitive and continuous monitoring of both volatile and nonvolatile CWAs, the National Research Institute of Police Science, in coordination with Hitachi Ltd., (based in Tokyo, Japan) has developed on-site detection methods utilizing counter-flow introduction (CFI) APCI MS (see Fig. 57.10). CFI-APCI-MS technology provides soft ionization of the suctioned CWAs by corona discharge (APCI) and introduction of only the produced primary target ions into the mass analyzer; the secondary interfering ions are excluded from the ionization region (CFI). The CFI-APCI technology is superior in terms of noise ion reduction, realizing ultrasensitive detection. Adopting an ion-trap mass analyzer (MS^n function), almost all types of CWAs, including vomit agents and lachrymators, are detected within several seconds with the LOD in the sub-μg/m^3 area (Seto et al., 2014; Okumura et al., 2015) (DS-1000; Fig. 57.11).

The monitoring tape method (Nakano and Nagashima, 2001) detects hazardous gases by spectrophotometrically measuring the color change on the tape or tab impregnated with specific reagents after reacting with the suctioned air sample (Fig. 57.12). The machines using this technology, developed by Riken Keiki Ltd., are commercially available. A diffusion-type apparatus is used as the portable machine, and by selecting the appropriate tab, the machine can monitor the desired gases. A transmission-type apparatus is used as the fixed monitor machine, and provides more sensitive detection. Riken Keiki and the author have developed a three tab-arrayed detector (FP-100), whereby three types of gaseous CWAs can be detected simultaneously and specifically with LODs lower than 1 mg/m^3 within 1 min. Blood agents (such as AC, CK, and arsine) and choking agents (such as CL and GC) and L1 can be detected.

57.7 Concluding remarks and future directions

A recommended on-site detection system for considering on-site CWA detection requirements and the status of present detection technologies is shown in Fig. 57.13. For portable detectors utilized by first responders, a combination of IMS instrument, a machine employing the arrayed monitoring tape method, and an electrochemical sensor is desirable. This combination encompasses the detection of

TABLE 57.6 Comparison of detection performances of various chemical warfare agent detectors.

	Gaseous agent	Nerve gas	Blister agent	Vomit agent / Lachrymator	Interference	Response time	Recovery time	Operation
Gas detection tube	OK[a]	OK	OK	X[a]	Δ[a]	X	–	X
	1 mg/m^3	0.3–2 mg/m^3	0.01–2 mg/m^3			1–7 min		
IMS aspiration	X	OK	OK	X	Δ	OK	OK	OK
	>300 mg/m^3	0.1–0.10 mg/m^3	1–10 mg/m^3			7–11 s	seconds–minutes	portable
IMS short drift	Δ	OK	OK	Δ	Δ	OK	OK	OK
	10–500 mg/m^3	0.3 mg/m^3	5–10 mg/m^3			3–20 s	seconds–minutes	portable
	0.06–15 mg/m^3[b]							
IMS	OK	OK	OK	Δ	Δ	OK	OK	OK
Long drift	0.1–10 mg/m^3	0.01–10 mg/m^3	0.1–20 mg/m^3			3–30 s	seconds–minutes	portable
FPD	X	OK	Δ (nonAs)	Δ	Δ	OK	OK	OK
	ND	0.1 mg/m^3	1 mg/m^3			2–5 s	seconds	portable
PID	Δ	X	Δ	Δ	X	Δ	Δ	OK
	100 mg/m^3	100 mg/m^3	100 mg/m^3			5–10 s	seconds	portable
SAW	Δ	X	Δ	Δ	Δ	Δ	X	OK
	50 mg/m^3	50 mg/m^3	100 mg/m^3			5–13 s	4–5 min	portable
FT/IR multiple reflection	Δ	OK	OK	Δ	Δ	OK	OK	Δ
	1 mg/m^3	0.1 mg/m^3	0.1 mg/m^3			10 s	–	portable
GC-FID	X	OK	OK	Δ	Δ	X	Δ	Δ
						5–10 min	minutes	portable
GC with trap	X	OK	OK	Δ	OK	X	Δ	Δ fixed
GC-MS with trap	Δ	OK	OK	Δ	OK	X	Δ	Δ
	1–400 mg/m^3[c]	0.1–2[d] mg/m^3	0.1 mg/m^3			10–15 min	minutes	portable
MS EI	X	Δ	Δ	X	Δ	OK	OK	OK
						minutes	minutes	portable

The values depicted are limits of alarm and respective times experimentally measured by the author's group.
[a]*OK: acceptable; Δ: improvement is required; X: not acceptable.*
[b]*Ionization: atmospheric electron emission.*
[c]*Direct gas analysis bypassed of GC separation.*
[d]*VX and RVX detection using VX-G conversion tube.*

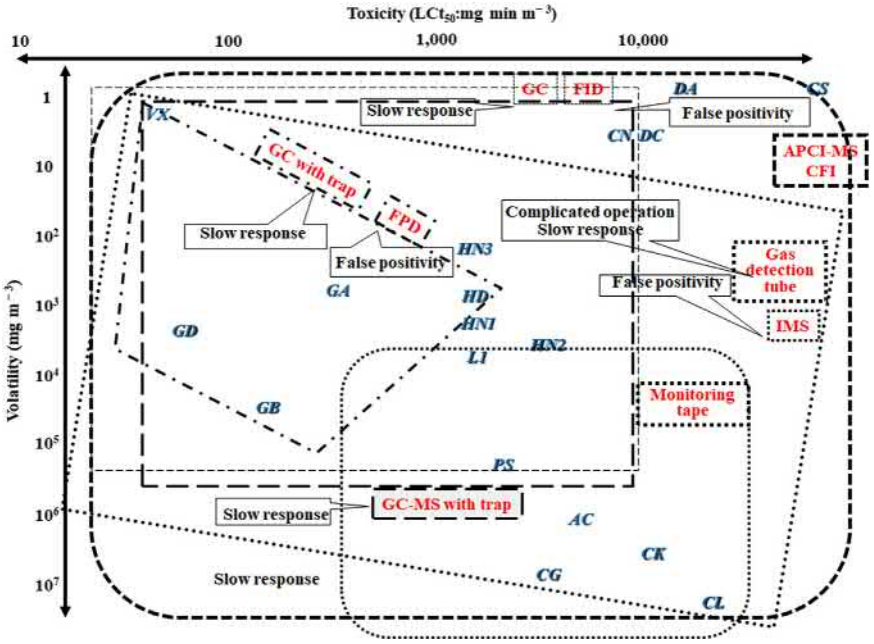

FIGURE 57.10 Performance of on-site CWA detection equipment in terms of target toxicities and volatilities. CWAs are shown in italics. The acceptable detection boundaries achieved for appropriate technologies are shown. Particular drawbacks for certain technologies are also described. *Reproduced with permission from Seto, Y., Sekiguchi, H., Maruko, H., et al., 2014. Sensitive and comprehensive detection of chemical warfare agents in air by atmospheric pressure chemical ionization ion trap tandem mass spectrometry with counter-flow introduction. Anal. Chem. 86, 4316–4326.*

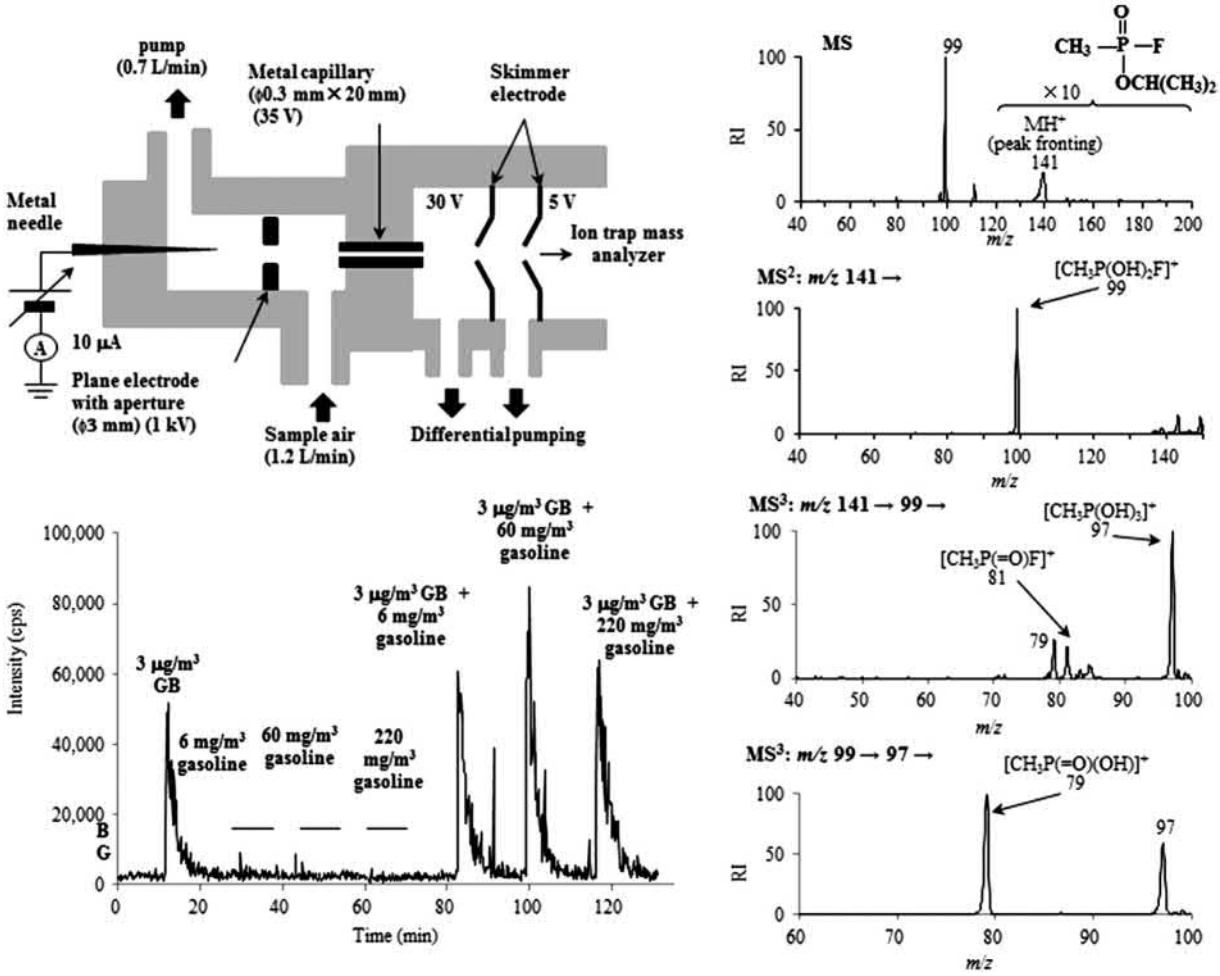

FIGURE 57.11 Counter-flow introduction/atmospheric pressure chemical ionization mass spectrometer. (Left upper) Structure of ionization chamber; (left lower) signal time response for sarin by MS^3 ($MH^+ \rightarrow m/z$ 99 $\rightarrow m/z$ 97) for sample air introduction with and without sarin and gasoline vapors; (right) MS^n mass spectra for sarin.

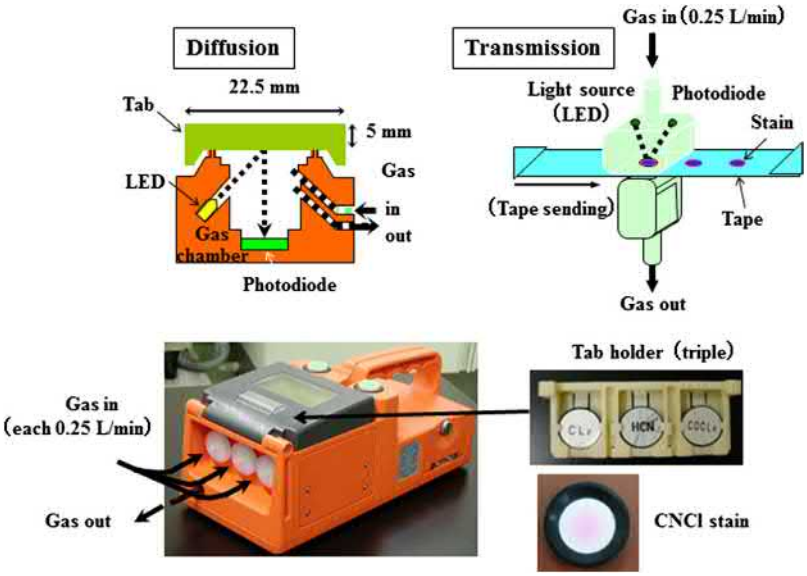

FIGURE 57.12 Monitoring tape method. Schematic detection mechanism of diffusion (upper left) and transmission (upper right) types of apparatus and arrayed diffusion-type detector (lower).

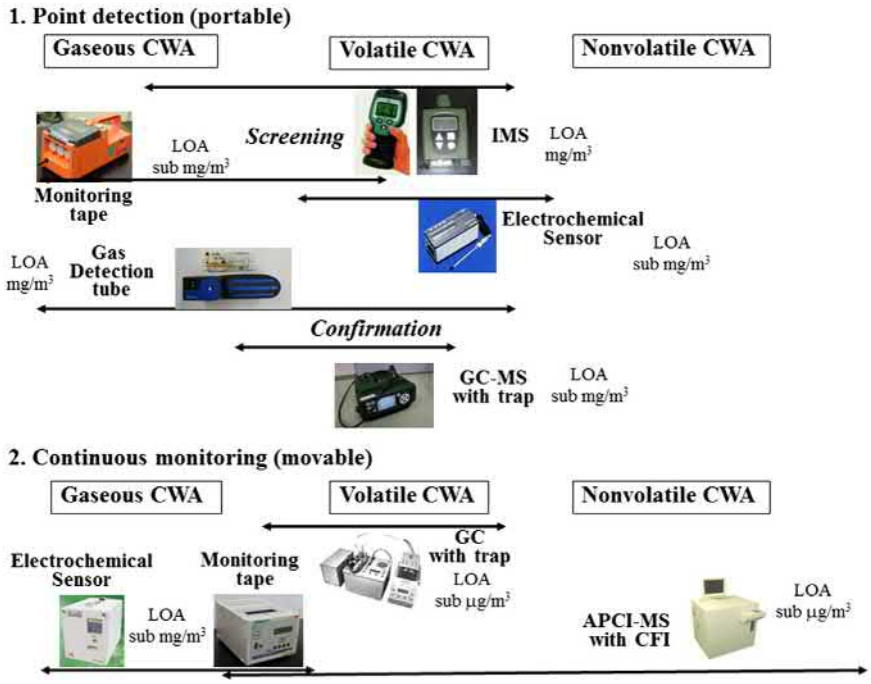

FIGURE 57.13 Recommended combinations of on-site detection equipment for point detection and continuous monitoring equipment for gaseous CWAs (blood agents, choking agents), volatile CWAs (nerve agents, blistering agents), and nonvolatile CWAs (vomiting agents, lachrymators). The acceptable detection boundaries for the technologies are shown, along with the LOA values.

CWA forms from the gaseous to the volatile state. GC-MS and gas detection tubes further assist in identifying the detected CWAs. Nonvolatile CWAs are still out of the detection range of portable machines. For movable detectors, highly sensitive detection equipment is necessary; the combination of CFI-APCI-MS and a lined set of machines using the transmission-type monitoring tape method or electrochemical sensor is almost ideal, handling all the CWAs with the required sensitivity. It is anticipated that new sensing technologies will be developed in the future that will overcome the problems of large size, false positives, low sensitivity, and narrow range of detection.

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Chapter 58

Neuropathy target esterase as a biomarker and biosensor of delayed neuropathic agents

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58.1 Introduction

Delayed neuropathic (DN) agents represent a new functional class of potential organophosphorus (OP) threats that have heretofore not been used in warfare or terrorist acts. Nevertheless, efforts should continue for the further development of faster methods for specific detection of DN agents in the environment and in biological samples (Kurochkin et al., 2009; Makhaeva et al., 2009). Moreover, it is vital to develop effective medical countermeasures against DN agents for at least four reasons: (1) These chemicals can readily be produced by rather straightforward modifications of the structures of conventional nerve agents or OP insecticides. (2) Warning signs and symptoms of toxicity may be lacking until 1−4 weeks after exposure, when the permanent sensory deficits and paralysis associated with OP compound−induced delayed neurotoxicity (OPIDN) ensue. (3) There are no established means of preventing or treating OPIDN. (4) A successful attack on a population with DN agents would produce an epidemic of debilitating neurological illness. In addition to the damaging effects of the disease itself, such an event would precipitate widespread fear and alarm akin to that of the polio epidemics of the 1950s and as recently seen on a much smaller scale with outbreaks of the polio-like illness, acute flaccid myelitis (Cartwright and Wessol, 2019).

This chapter begins with a review of current understanding about the chemistry and mechanism of action of DN agents. It then describes how existing knowledge can be applied to generate biomarkers and biosensors for discriminating between conventional and DN agents. The chapter ends by indicating future work needed to address the potential threat of DN agents and the promise of NTE and OPIDN research for understanding, preventing, and treating other neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS).

58.2 Organophosphorus compounds

58.2.1 Conventional nerve agents versus delayed neuropathic agents

Conventional nerve agents, such as sarin and soman, are OP compounds of pentavalent phosphorus that are well known as threats in the context of warfare and terrorism (Sidell and Borak, 1992). These substances produce acute cholinergic toxicity and death via inhibition of acetylcholinesterase (AChE) in the central and peripheral nervous systems (Marrs et al., 1996).

Unlike conventional nerve agents, DN agents produce permanent neurological dysfunction in the form of OPIDN rather than cholinergic toxicity and death (Richardson and Makhaeva, 2014; Richardson et al., 2013). Like conventional nerve agents, DN agents are OP compounds of pentavalent phosphorus that are readily synthesized, but they are designed to inactivate neuropathy target esterase

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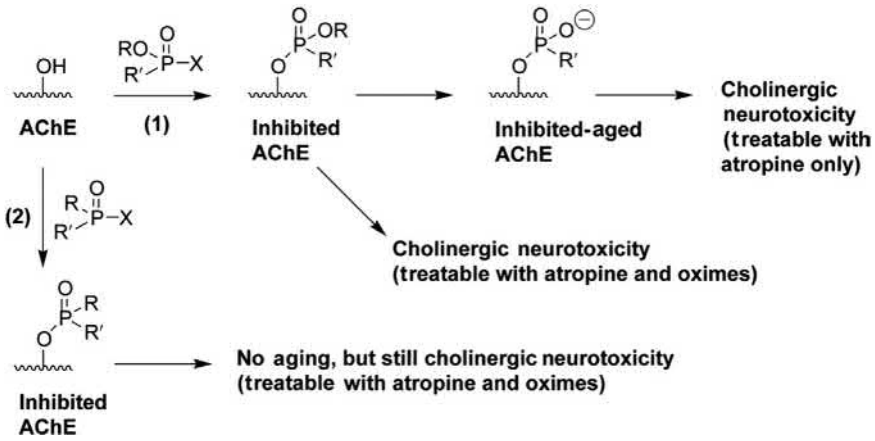

FIGURE 58.1 Reaction of acetylcholinesterase (AChE) with an organophosphonate in pathway (1) yields a phosphonylated (inhibited) enzyme, which can undergo net loss of an R-group to yield an inhibited-aged enzyme. Inhibition alone produces cholinergic toxicity that is treatable with both atropine to counteract excess acetylcholine at muscarinic acetylcholine receptors and oximes to reactivate AChE. Aging does not change the type of toxicity, but it renders the enzyme intractable to reactivation, so that treatment with oximes becomes ineffective. Reaction of AChE with an organophosphinate in pathway (2) yields a phosphinylated (inhibited) enzyme and produces cholinergic toxicity, but because of the stability of the C−P bonds linking the R- and R′-groups to phosphorus, the phosphinyl moiety on AChE does not undergo aging, so that both atropine and oximes can be used in therapy. R, R′, substituted or unsubstituted alkyl or aryl groups, which can be different or equivalent. X, primary leaving group, for example, fluoride. *Reproduced with permission from Richardson, R.J., Makhaeva, G.F., 2014. Organophosphorus compounds. In: P. Wexler (Ed.), Encyclopedia of Toxicology, third ed., vol. 3. Elsevier, Ltd., Oxford, pp. 714−719.*

(neurotoxic esterase, NTE) in preference to AChE (Makhaeva et al., 2012, 2013; Wu and Casida, 1995). Because of this selectivity, DN agents may elicit little or no warning signs of acute cholinergic toxicity, so that victims of DN agents might not know they have been exposed until OPIDN develops 1−4 weeks later.

Cholinergic toxicity from OP compounds can be elicited solely by inhibition of AChE (Richardson, 2018). However, the AChE−OP conjugate can undergo an additional reaction ("aging") entailing loss of an OP ligand (side chain or R-group) to yield a negatively charged phosphoryl adduct on the active site serine. Although the aging reaction of organophosphylated AChE has practical implications for therapy, it does not change the qualitative nature of the toxicity (Fig. 58.1).

In contrast to the situation with AChE inhibitors and cholinergic toxicity, decades of toxicological research have shown that inhibition of NTE on its own is apparently insufficient to produce OPIDN. Inhibition is the obligatory first step, but it appears that this must be followed by aging of the NTE−OP conjugate in order to trigger axonal degeneration (Fig. 58.2) (Moser et al., 2019; Richardson and Makhaeva, 2014; Richardson et al., 2013).

Consequently, interactions of NTE with DN agents certainly result first in a loss of physiological function of enzymatic activity. However, the seeming dependence of OPIDN on the aging reaction implies a gain of toxic function resulting from a particular chemical modification of the protein rather than, or in addition to, loss of NTE activity. However, neurodegenerative diseases can involve both processes, as exemplified by loss of normal function through protein misfolding and gain of toxic function via the deleterious action of the misfolded proteins (Winklhofer et al., 2008).

Thus, DN agents are distinct from conventional nerve agents in several respects. Furthermore, especially in view of their ease of synthesis, absence of initial signs or symptoms of exposure, and lack of prophylactic or therapeutic measures, it is conceivable that rogue nations or terrorist groups might consider DN agents attractive as weapons of permanent incapacitation against military and/or civilian populations. Therefore, part of an effective chemical defense strategy is to develop methods for detecting DN agents and distinguishing them from conventional nerve agents. This can be done by exploiting the sensitivity and selectivity afforded by appropriate biomarkers and biosensors (Kohli et al., 2007a, 2010, 2014; Makhaeva et al., 2003; Malygin et al., 2003; Srivastava et al., 2010; Sigolaeva et al., 2010, 2013).

58.2.2 Organophosphorus compounds of pentavalent versus trivalent phosphorus

In this chapter, DN agents are defined as OP compounds of pentavalent phosphorus that produce OPIDN. Examples shown in Fig. 58.3 are ethyl *n*-octylphosphonofluoridate (EOPF) and 2-(2-methylphenoxy)-4H-l,3,2-benzodioxaphosphorin-2-oxide, abbreviated as [2-(2-methylphenoxy)-BDPO] or CBDP, the active metabolite of the archetypal DN agent, tri-*o*-cresyl phosphate (TOCP) (Wu and Casida, 1995). TOCP was the causative agent of major outbreaks of OPIDN affecting over

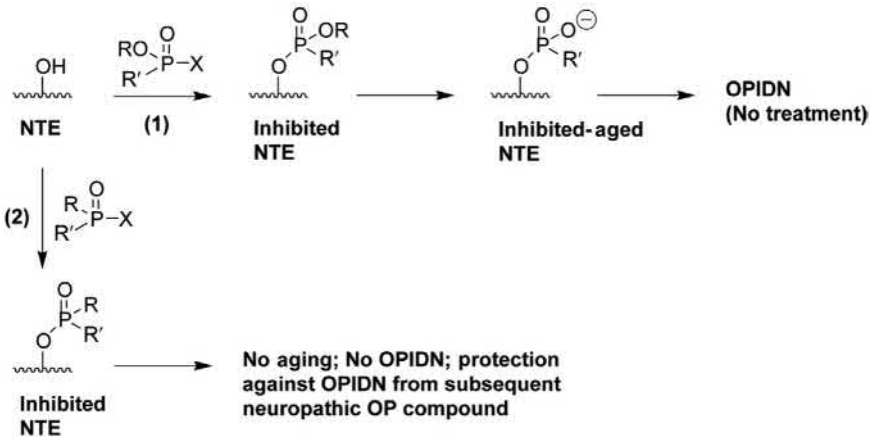

FIGURE 58.2 Reaction of neuropathy target esterase (NTE) with an organophosphonate in pathway (1) yields a phosphonylated (inhibited) enzyme, which can undergo net loss of an R-group to yield an inhibited-aged enzyme. Inhibition alone does not produce organophosphorus (OP) compound-induced delayed neurotoxicity (OPIDN); both inhibition and aging are required, and there is no treatment for the neuropathy. Reaction of NTE with an organophosphinate in pathway (2) yields a phosphinylated (inhibited) enzyme, but because of the stability of the C−P bonds linking the R- and R′-groups to phosphorus, the phosphinyl moiety on NTE does not undergo aging, so that OPIDN does not occur. Inhibition of NTE with a nonaging inhibitor is not biologically inert—it protects against subsequent exposures to neuropathic (ageable) NTE inhibitors. R, R′, substituted or unsubstituted alkyl or aryl groups, which can be different or equivalent. X, primary leaving group, for example, fluoride. *Reproduced with permission from Richardson, R.J., Makhaeva, G.F., 2014. Organophosphorus compounds. In: P. Wexler (Ed.), Encyclopedia of Toxicology, third ed., vol. 3. Elsevier, Ltd., Oxford, pp. 714−719.*

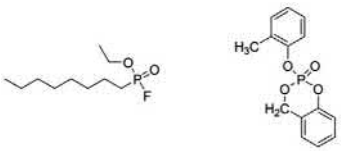

FIGURE 58.3 Examples of delayed neuropathic (DN) agents containing pentavalent phosphorus: ethyl *n*-octylphosphonofluoridate (EOPF) and 2-(2-methylphenoxy)-4H-l,3,2-benzodioxaphosphorin-2-oxide [2-(2-methylphenoxy)-BDPO or CBDP], the active metabolite of the archetypal DN agent, tri-*o*-cresyl phosphate (TOCP) (Wu and Casida, 1995).

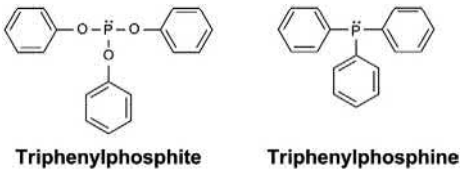

FIGURE 58.4 Examples of neuropathic organophosphorus compounds containing trivalent phosphorus: triphenylphosphite and triphenylphosphine (Eto, 1974).

70,000 people during the past ∼90 years (Johnson and Glynn, 2001); it has also been implicated recently in the etiology of aerotoxic syndrome (Heutelbeck et al., 2016; Schopfer et al., 2014) as well as ALS and other neurodegenerative diseases (Merwin et al., 2017).

However, there are also OP compounds of trivalent phosphorus that produce neurological injury. Examples shown in Fig. 58.4 are triphenylphosphite and triphenylphosphine. Comparatively little is known about the neuropathic trivalent OP compounds, but the clinical course and spatial−temporal pattern of lesions that they produce vary from OPIDN (Lehning et al., 1996), and the mechanisms of action of these compounds appear to differ from that of the DN agents. For example, triphenylphosphite produces a syndrome of delayed axonopathy and other neurodegenerative changes that could represent a variant of OPIDN, possibly via inhibition/aging of NTE (Abou-Donia, 1992; Fioroni et al., 1995; Padilla et al., 1987; Tanaka et al., 1990; Carlson and Ehrich, 2004). However, although triphenylphosphite inhibits NTE in vitro and in vivo, the nature of the adduct formed with NTE has not been elucidated, and the aging reaction of triphenylphosphite-inhibited NTE has apparently not been studied. Triphenylphosphine is another trivalent OP compound that produces delayed axonopathy, but its pathogenesis and mechanism appear to differ from those of triphenylphosphite or neuropathic OP compounds of pentavalent phosphorus. Among other differences, triphenylphosphine does not inhibit NTE in vitro or in vivo (Davis et al., 1999; Padilla et al., 1987).

With the foregoing in mind, the remainder of this chapter will deal exclusively with OP compounds of pentavalent phosphorus.

58.3 Organophosphorus compound−induced delayed neurotoxicity

OPIDN, also known as OP compound−induced delayed polyneuropathy (OPIDP), can be produced within 1−4

weeks of a single exposure to a DN agent. With compounds that are highly selective for NTE, the first week of this period can be clinically quiescent (Davis and Richardson, 1980; Lotti, 1992; Lotti and Moretto, 2005; Richardson and Makhaeva, 2014; Richardson et al., 2013). Pathogenesis involves progressive distal degeneration of sensory and motor axons in peripheral nerves and spinal cord tracts. Accordingly, symptoms begin with paresthesias in the distal extremities followed by sensory loss, ataxia, and flaccid paralysis. Once the disease is initiated, it advances inexorably. In keeping with its unknown mechanism of neurodegeneration, there is no cure or treatment other than general supportive therapy. Over a period of months to years, some regeneration of peripheral nerve axons may occur to reinnervate muscle. Nevertheless, the concomitant persistence of injury to descending inhibitory pathways in the spinal cord prevents complete functional recovery and permits only a shift from flaccid to spastic paralysis (Richardson and Makhaeva, 2014; Richardson et al., 2013).

Because of the insidious onset and permanent debilitating effects of OPIDN, it is essential to be able to predict the potential of a given OP compound to produce this disease as opposed to causing acute cholinergic toxicity. Furthermore, it is important to develop specific and stable biomarkers and biosensors of exposure to DN agents and devise countermeasures against them (Kohli et al., 2007a; Makhaeva et al., 2003; Malygin et al., 2003; Sigolaeva et al., 2010, 2013). To accomplish these goals, it will be necessary to acquire a level of mechanistic understanding of interactions between DN compounds and target macromolecules akin to what has been achieved for conventional (anti-AChE) nerve agents. Although there is much left to be learned about the pathogenesis and mechanism of OPIDN, sufficient knowledge exists about the apparent initiating events, that is, inhibition and aging of NTE, to provide a foundation for the development of biomarkers and biosensors of DN agents.

58.4 Neuropathy target esterase

58.4.1 Definition of neuropathy target esterase and its potential normal or pathogenic roles

NTE (UniProtKB/Swiss-Prot Q81Y17; PLPL6_HUMAN) has been categorized as a lysophospholipase (EC 3.1.1.5), owing to its ability to hydrolyze phosphatidylcholine to glycerophosphocholine (UniProt, 2019). It is also known as patatin-like phospholipase domain-containing protein 6, whose gene name is PNPLA6, because the catalytic domain of NTE encompasses a region with sequence homology to patatin, a phospholipase found in potatoes and other plants. Thus, it appears that NTE can function biochemically as a phospholipase, but its precise physiological role remains to be firmly established (Glynn, 2013; Richardson et al., 2013; Wijeyesakere and Richardson, 2010).

The human PNPLA6 gene encoding NTE has 37 exons and 25 transcripts (Ensembl, 2019). Thus, it is not surprising that the UniProt entry for human NTE lists four protein isoforms attributed to alternative splicing along with 10 computationally mapped potential isoforms (UniProt, 2019). Although UniProt has adopted isoform-4 with 1375 amino acid residues as the canonical sequence, this chapter defines human NTE as isoform-2, which is the sequence initially reported by Lush et al. (1998), consisting of 1327 amino acids and having a molecular weight without post-translational modifications of 146 kDa. The protein has a transmembrane domain near the *N*-terminus (residues 9−31) and three tandem putative cyclic nucleotide binding domains (residues 163−262, 480−573, and 597−689), in addition to the patatin domain (residues 933−1099), which contains the catalytic active site (Wijeyesakere et al., 2007; Wijeyesakere and Richardson, 2010).

The three-dimensional structure of NTE has not been experimentally determined, but a homology model of the patatin domain indicates that the active site serine (Ser^{966}) is located on a "nucleophilic elbow" characteristic of serine hydrolases (Wijeyesakere et al., 2007). Moreover, the model indicates that the catalytic site of NTE consists of a novel Ser-Asp catalytic dyad, as in patatin and mammalian cytosolic phospholipase A2 ($cPLA_2$), rather than the classical catalytic triad (Ser-Asp/Glu-His), as found in many serine hydrolases including AChE (Dodson and Wlodawer, 1998).

Mutations have been identified in NTE that are associated with a form of motor neuron disease (NTE-MND) (Rainier et al., 2008, 2011). Constructs containing NTE-MND mutations within the catalytic domain result in expressing NTE protein that has altered inhibition and aging kinetics (Hein et al., 2010a). In addition, cultured fibroblasts from human subjects with NTE-MND exhibit decreased NTE catalytic activity (Hein et al., 2010b). However, although fibroblasts from human subjects heterozygous for an insertion mutation that was expected to truncate the NTE protein had markedly reduced NTE activity (40%−43% of control), these subjects were asymptomatic, indicating that decreased NTE activity alone was insufficient to produce disease.

More recently, additional human NTE mutations have been cataloged that exhibit a variety of disease phenotypes, including spastic paraplegia, spinocerebellar degeneration, and photoreceptor degeneration resulting in blindness (Hufnagel et al., 2015; Kmoch et al., 2015; Topaloglu et al., 2014).

NTE was first identified as the presumptive target of neuropathic OP compounds in the initiation of OPIDN (Johnson, 1970). Its activity in cells and tissues is operationally defined as the enzymatic hydrolysis of the

nonphysiological substrate, phenyl valerate, which is resistant to inhibition by diethyl 4-nitrophenyl phosphate (paraoxon) and sensitive to inhibition by *N,N′*-diisopropylphosphorodiamidic fluoride (mipafox) under specified conditions of preincubation with inhibitors and subsequent incubation with substrate (Johnson, 1977; Kayyali et al., 1991; Makhaeva et al., 2007).

Because the full-length protein is difficult to isolate or produce, human recombinant *N*TE *est*erase domain (NEST) has been used as an NTE surrogate for studies in vitro that require pure protein rather than a crude preparation containing NTE (Kropp et al., 2004). NEST comprises residues 727−1216 of NTE; it has been shown to be the shortest segment of NTE that retains esterase activity. Moreover, the catalytic properties of NEST, including its response to OP inhibitors, closely resemble those of full-length NTE (Atkins and Glynn, 2000; Atkins et al., 2002; Kropp et al., 2004; Van Tienhoven et al., 2002). The catalytic activity of NEST has been shown to alter the fluidity of bilayer lipid membranes (Greiner et al., 2010), and NEST has been incorporated into a biosensor for the detection of DN agents (Kohli et al., 2007a, 2014).

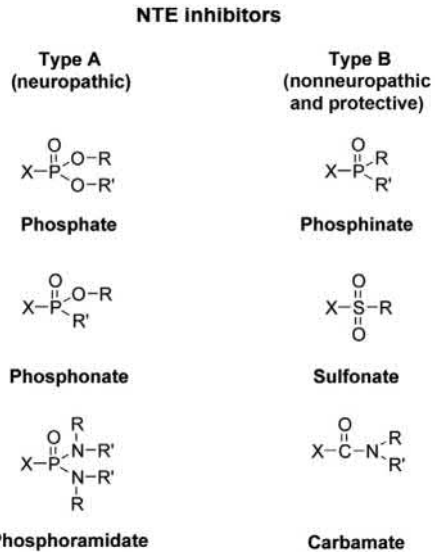

FIGURE 58.5 Subclasses of neuropathy target esterase (NTE) inhibitors. Type A NTE inhibitors include phosphates, phosphonates, and phosphoramidates; these are neuropathic and capable of aging. Type B NTE inhibitors include phosphinates, sulfonates, and carbamates; these are non-neuropathic and not capable of aging. However, inhibition of NTE with a type B inhibitor will protect against organophosphorus compound-induced delayed neurotoxicity (OPIDN) from subsequently administered type A inhibitors. *Reproduced with permission from Richardson, R.J., Makhaeva, G.F., 2014. Organophosphorus compounds. In: P. Wexler (Ed.), Encyclopedia of Toxicology, third ed., vol. 3. Elsevier, Ltd., Oxford, pp. 714−719.*

58.4.2 Role of neuropathy target esterase in organophosphorus compound−induced delayed neurotoxicity

Early studies indicated that the initial molecular events in OPIDN are the concerted inhibition and aging of a threshold level (>70%) of NTE in the central and peripheral nervous systems (Glynn, 1999; Johnson, 1974, 1982; Lotti, 1992). As with other serine esterases, inhibition of NTE is thought to occur by nucleophilic attack of the active site serine (Ser966) at the phosphorus of the OP compound, with displacement of a primary leaving group. Aging of the phosphylated enzyme presumably involves loss of a second ligand, leaving a negatively charged phosphyl moiety covalently attached to the active site. Note that "phosphyl" is used as a generic term for the various types of OP compounds that might be used as inhibitors of serine esterases, for example, phosphates, phosphonates, phosphinates, and phosphoramidates.

The apparent necessity for aging of NTE as a trigger for OPIDN came about from analyses of structure−activity relationships. These studies revealed that NTE inhibitors fall into two functional categories, types A and B (Davis and Richardson, 1980; Davis et al., 1985; Johnson, 1975) (Fig. 58.5).

Type A NTE inhibitors produce OPIDN and include certain phosphates, phosphonates, and phosphoramidates. When type A compounds inactivate NTE, it rapidly becomes intractable to reactivation by powerful nucleophiles, for example, oximes or fluoride. This inability to be reactivated has been interpreted as evidence of aging. Because loss of reactivatability takes place within minutes of inhibition by type A inhibitors and OPIDN takes 1−4 weeks to develop, aging of NTE is not the rate-limiting step in the disease process (Johnson, 1982).

Type B NTE inhibitors do not produce OPIDN and include certain phosphinates, carbamates, and sulfonates. NTE conjugates with type B compounds are considered not to undergo aging; those formed from carbamates and phosphinates can be reactivated; moreover, carbamylated, phosphinylated, or sulfonylated NTE cannot undergo further reactions to produce a stable charged species. Despite the fact that inhibition of NTE by type B compounds does not result in OPIDN, this reaction is far from being biologically inert. Pretreatment of animals with type B inhibitors protects them against OPIDN from subsequently administered type A inhibitors, presumably by blocking the formation of aged NTE (Davis et al., 1985; Johnson, 1990; Richardson, 1984) (Fig. 58.2). Protection against type A inhibitors by type B inhibitors is effective as long as inhibition of NTE by type B compounds is >30%, thus preventing inhibition and aging of the >70% of NTE required for induction of OPIDN.

It would appear that the ostensible requirement for aging of NTE in the development of OPIDN is not merely

to ensure irreversible inhibition of the enzyme. Prolonged suprathreshold inhibition (>70%) of neural NTE in vivo by repeated dosing of nonaging inhibitors does not cause neuropathy (Johnson, 1970, 1974, 1982). Moreover, repeated low-level dosing with aging inhibitors to produce a prolonged steady-state inhibition that is below threshold (<70%) does not result in OPIDN until a higher single dose is superimposed that raises the inhibition level above threshold. In the latter scenario, there is still a delay of 10–14 days from the high point of inhibition (and presumed concomitant aging) until signs of neuropathy appear (Johnson and Lotti, 1980; Lotti and Johnson, 1980). These results suggest that simple loss of catalytic activity of NTE is not the damaging event in OPIDN. Thus, the notion has arisen that the "biochemical lesion" that triggers OPIDN is the formation of a chemically modified (aged) protein that has gained a toxic function in the neuron (Glynn, 2000; Richardson, 1984; Richardson et al., 2013).

The pathogenic role of aged NTE is not known, but it is tempting to speculate that it may be involved in an axonal self-destruct program that has been proposed to be an important mechanism in neurodegenerative disorders, for example, motor neuron disease (Raff et al., 2002). Such a program might be triggered by chemically modified NTE acting through a gain of toxic function mechanism. On the other hand, experiments with conventional NTE knockouts (Moser et al., 2004; Mühlig-Versen et al., 2005; Winrow et al., 2003) and conditional NTE knockouts (Akassoglou et al., 2004; Read et al., 2010), along with demonstrations of a likely role of NTE in membrane lipid metabolism (Glynn, 2013; Quistad et al., 2003; Van Tienhoven et al., 2002; Zaccheo et al., 2004; Zhu et al., 2016) and endoplasmic reticulum stress (Sunderhaus et al., 2019), indicate that lethality or neuropathology can result from a loss of function of NTE (Glynn, 2006). However, the two possibilities—gain of toxic function and loss of physiological function—are not necessarily mutually exclusive and can in fact coexist in various neurodegenerative diseases (Winklhofer et al., 2008). In any event, while the physiological and pathogenic roles of NTE are being deciphered, the fact that an excellent correlation exists between inhibition/aging of NTE and induction of OPIDN is sufficient to enable the pragmatic use of this information for the development of biomarkers and biosensors for DN agents (Makhaeva et al., 2003, 2007).

58.5 Kinetics of organophosphorus inhibitor–serine hydrolase interactions

58.5.1 Introduction

The inhibitory and postinhibitory steps in the interaction of an OP compound with a serine hydrolase (E-OH), such as NTE or AChE, are illustrated in Fig. 58.6. The mathematical relationships describing the kinetics of irreversible inhibition of serine esterases by OP compounds and postinhibitory reactions of the enzyme–inhibitor adduct (conjugate) summarized here were elegantly set forth in the classic work by Aldridge and Reiner (1972), and synopses are available in other sources (Clothier et al., 1981; Main, 1980; Richardson, 1992). The equations featured below provide a basic foundation for determining the inhibitory potency of OP compounds against serine hydrolases as well as the rates of reactivation or aging of the inhibited hydrolases. Such approaches can be employed to assess the neuropathic potential of compounds and to distinguish conventional nerve agents from DN agents using biomarkers and biosensors. For more complex scenarios beyond the scope of this chapter, other equations have been developed (Estevez and Vilanova, 2009).

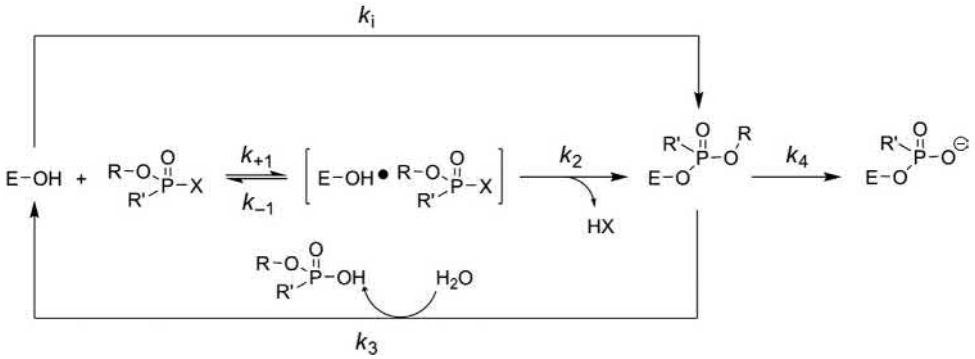

FIGURE 58.6 Reactions between a serine hydrolase and an organophosphonate inhibitor showing associated rate constants. The initial reversible reaction with forward (k_{+1}) and back (k_{-1}) rate constants forms a Michaelis-type complex. The complex can undergo phosphonylation (rate constant k_2) with expulsion of the primary leaving group, X. The overall progress of the reaction from enzyme to phosphonylated (inhibited) product is characterized by the bimolecular rate constant of inhibition, k_i. The phosphonylated enzyme can undergo spontaneous reactivation (hydrolysis in the presence of excess water) described by the pseudo-first-order rate constant, k_3, or undergo net loss of the R-group (aging) characterized by the first-order rate constant, k_4 (Richardson, 1992).

58.5.2 Inhibition

The first step leading to E-OH inhibition is a reversible association of the OP compound (depicted in Fig. 58.6 as a phosphonate) and enzyme to form a Michaelis-type complex (shown in square brackets); the associated rate constants for the forward (k_{+1}) and reverse (k_{-1}) reactions are shown on the double arrow. The active site serine hydroxyl of the hydrolase then engages in a nucleophilic attack on the phosphorus atom of the OP inhibitor, displacing the primary leaving group ($X-$), resulting in a phosphonylated enzyme with net loss of HX. The primary leaving group, $X-$, is typically a conjugate base of a strong acid, HX, such as HF. The phosphonylation rate constant is shown as k_2 (Richardson, 1992).

Considering only the first two steps in the reaction sequence, which is appropriate for most acylating inhibitors, the affinity of the inhibitor for the enzyme is given by the Michaelis-type equilibrium constant, K_d, as shown in Eq. (58.1). Note that Aldridge and Reiner (1972) denote this constant by K_a, while here, we use K_d, according to the terminology of Main (1980):

$$K_d = \frac{k_{-1} + k_2}{k_{+1}} \tag{58.1}$$

The overall progress of the reaction from E-OH and inhibitor to phosphonylated enzyme is characterized by the bimolecular rate constant of inhibition, k_i. This important measure of inhibitory potency is determined by measuring the activity remaining as a function of time of preincubation with various concentrations of inhibitor. The substrate is added after the preincubation interval (Richardson, 1992). When the inhibitor concentration $[OP] << K_d$, k_i is given by Eq. (58.2).

$$k_i = \frac{k_2}{K_d} \tag{58.2}$$

So-called ideal first-order kinetics of inhibition are obtained under the commonly observed conditions when the concentration of the Michaelis-type complex is small, k_2 is high, k_3 is much smaller than k_2, and $[OP] > 10[E\text{-}OH]$, where [E-OH] = concentration of native enzyme. In such cases we have Eq. (58.3).

$$\ln\left(\frac{v}{v_0}\right) = \frac{k_2[OP]t}{[OP] + K_d} \tag{58.3}$$

In Eq. (58.3), v is the rate of enzymatic hydrolysis of its substrate at time $= t$, and v_0 is the rate at time zero. Substituting (% activity/100) for (v/v_0) and letting $k' = k_2[OP]/([OP] + K_d)$ yields Eq. (58.4).

$$\ln\left(\frac{\%\ \text{activity}}{100}\right) = -k't \tag{58.4}$$

Thus, primary plots of ln(% activity/100) versus t will be straight lines with slopes $= -k'$ as shown in Fig. 58.7A. In addition, the experimentally determined dependence of % (activity remaining) on the preincubation time (t) and inhibitor concentration [OP] is given by Eq. (58.5).

$$\ln\frac{\%\ \text{activity}}{100} = -k_i[OP]t \tag{58.5}$$

Setting Eqs. (58.4) and (58.5) equal to each other gives Eq. (58.6).

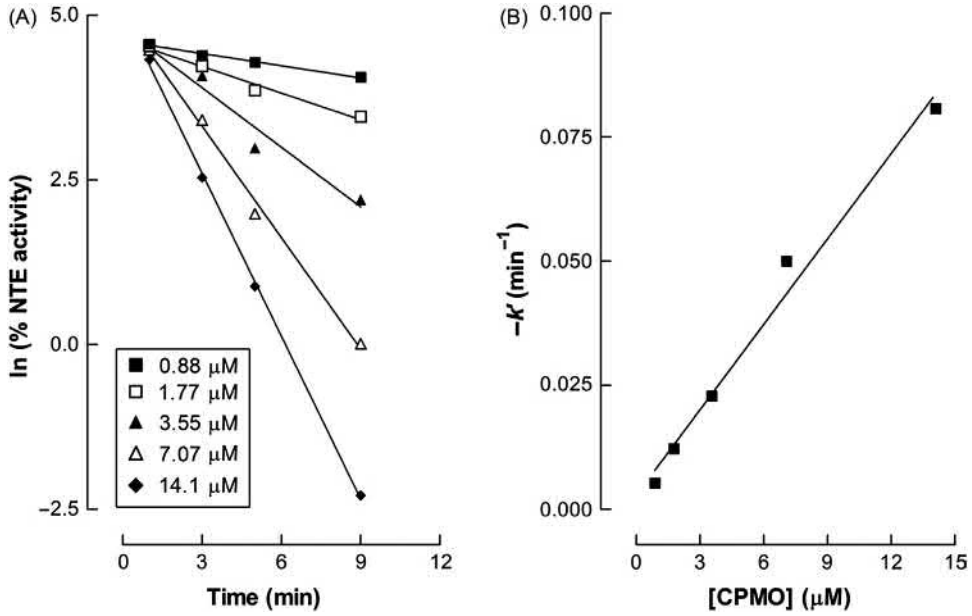

FIGURE 58.7 (A) Primary kinetic plots of ln(% NTE activity) versus time for various indicated concentrations of chlorpyrifos methyl oxon (CPMO). (B) Slopes ($-k'$) of primary kinetic plots versus inhibitor concentration. Data are for hen brain microsomal NTE at pH 8.0 and 37°C. *Reproduced with permission from Kropp, T.J., Richardson, R.J., 2003. Relative inhibitory potencies of chlorpyrifos oxon, chlorpyrifos methyl oxon, and mipafox for acetylcholinesterase versus neuropathy target esterase. J. Toxicol. Environ. Health Part A 66, 1145–1157.*

$$k' = k_i[\mathrm{OP}] \tag{58.6}$$

Therefore, a secondary plot of $-k'$ versus [OP] will yield a straight line with slope $= k_i$ as shown in Fig. 58.7B. The k_i value thus obtained is an indication of the inhibitory potency of a given compound against a particular serine hydrolase. As stated above, k_i is a measure of the overall progress of the reaction between the inhibitor and enzyme to yield the organophosphylated product. However, it is important to realize as shown in Eq. (58.2) that k_i is a composite of K_d, which is an indication of the affinity of the inhibitor for the enzyme, and k_2, which is an indication of the rate of phosphylation of the enzyme by the inhibitor. Inspection of Eqs. (58.2) and(58.5) shows that the units of k_i are $[\mathrm{OP}]^{-1}t^{-1}$; for example, the k_i obtained for chlorpyrifos methyl oxon (CPMO) against hen brain microsomal NTE at pH 8.0 and 37°C (Fig. 58.7) is $5.82 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{min}^{-1}$ (Kropp and Richardson, 2003; Richardson, 1992).

Under certain conditions, it is possible to determine the K_d and k_2 components of k_i separately. For example, if the secondary plot is not linear, this is an indication of an appreciable concentration of a Michaelis-type complex. In this case, the K_d term must be included; by combining Eqs. (58.3) and(58.4) and rearranging, we have Eq. (58.7).

$$\frac{[\mathrm{OP}]}{k'} = \frac{K_d}{k_2} + \frac{[\mathrm{OP}]}{k_2} \tag{58.7}$$

Thus, a Wilkinson plot of $[\mathrm{OP}]/k'$ versus [OP] will yield a straight line with slope $1/k_2$, a y-intercept of K_d/k_2, and an x-intercept of K_d. Moreover, from Eq. (53.2), $k_i = k_2/K_d$; therefore, the reciprocal of the y-intercept gives k_i (Richardson, 1992).

An especially useful relationship is given by substituting a % activity of interest into Eq. (58.5) to yield the inhibitor concentration at a given time of preincubation with enzyme that would yield the particular percent activity. For example, when $[\mathrm{OP}]_{50} = I_{50} =$ the inhibitor concentration required to produce 50% inhibition of the enzyme at a given time, t, of incubation of enzyme and inhibitor at defined conditions of pH, temperature, and ionic strength before adding substrate, we have Eq. (58.8).

$$[\mathrm{OP}]_{50} = I_{50} = \ln 2/k_i t \approx 0.693/k_i t \tag{58.8}$$

Note that in Eq. (58.8) k_i and I_{50} are reciprocally related and that I_{50} is time dependent. It is valid to calculate an I_{50} from a k_i value when ideal kinetics are observed. However, it is not valid to calculate a k_i from an experimentally determined fixed-time I_{50}, because the I_{50} alone contains no information about the kinetic behavior of the inhibition reaction. In addition, if inhibitory potency is assessed by measuring fixed-time I_{50} values, it is essential to report the time of preincubation along with the concentration, because the I_{50} decreases as the preincubation time increases (Aldridge and Reiner, 1972; Clothier et al., 1981; Richardson, 1992). For example, using Eq. (58.8), the 20-min I_{50} for chlorpyrifos methyl oxon against hen brain microsomal NTE at pH 8.0 and 37°C may be calculated from the k_i given above to be $0.693/(5.82 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{min}^{-1})(20\ \mathrm{min}) = 5.95 \times 10^{-7}\ \mathrm{M} \approx 0.60\ \mu\mathrm{M}$ (Kropp and Richardson, 2003).

58.5.3 Reactivation

In Fig. 58.6, the phosphonylated enzyme can undergo spontaneous reactivation via hydrolysis to yield free enzyme and a phosphonic acid, with an associated pseudo-first-order rate constant, k_3 (the reactivator, water, is present in great excess). The spontaneous rate constant of reactivation, k_3, can be obtained from Eq. (58.9).

$$\ln\left(\frac{100}{\%\ \text{inhibition}}\right) = k_3 t \tag{58.9}$$

The % reactivation in a reactivation experiment is determined by Eq. (58.10).

$$\%\ \text{inhibition} = \left[\frac{A - A_t}{A - A_0}\right] \times 100 \tag{58.10}$$

In Eq. (58.10), A is the activity of the control, A_t is the activity of the inhibited enzyme at time t, and A_0 is the activity of the inhibited enzyme at $t = 0$. In practice, reactivation is studied by preincubating the enzyme with a concentration of inhibitor that rapidly produces essentially complete inhibition. The inhibitor and enzyme are then rapidly separated or the preparation is diluted sufficiently to prevent further appreciable inhibition of free enzyme, and the return of enzyme activity is measured at timed intervals. The slope (k_3) of the least-squares best-fit line from a plot of ln(100/% inhibition) versus t is determined using linear regression from the initial linear portion of the curve. From Eq. (58.9), it can be seen that the units of k_3 are t^{-1}, for example, min^{-1}, and that the half-life, $t_{1/2}$, the time required for 50% reactivation, can be determined from Eq. (58.11).

$$t_{1/2} = \ln 2/k_3 \approx 0.693/k_3 \tag{58.11}$$

The apparent half-life of reactivation can range from minutes to weeks, depending upon the enzyme, inhibitor, pH, and temperature. Reactivation can appear to be slow or nonexistent if aging has occurred, because the aged form of the phosphylated enzyme is stable to hydrolysis and will not reactivate (Jianmongkol et al., 1999; Richardson, 1992).

58.5.4 Aging

As shown in Fig. 58.6, the inhibited esterase can also undergo aging, for example, via net loss of a labile R-group to yield the negatively charged phosphonyl adduct on the active site serine of the enzyme. Aging is characterized by a first-order rate constant, k_4, and the operational definition of this reaction is the time-dependent loss of the ability to reactivate the inhibited enzyme by powerful nucleophiles, such as fluoride ion or oximes. The method of determining aging is similar to that for reactivation. Enzyme is preincubated with a concentration of inhibitor sufficient to produce essentially complete inhibition in a relatively short time. Aliquots are then treated with a reactivator such as fluoride or an oxime and the amount of activity relative to a nonreactivated sample is measured. The first-order rate constant of aging, k_4, can be determined from Eq. (58.12).

$$\ln\left[\frac{100}{\%\ \text{reactivation}}\right] = k_4 t \tag{58.12}$$

In an aging experiment, the % reactivation is determined by Eq. (58.13).

$$\%\ \text{reactivation} = \left[\frac{AR_1 - AI_1}{AR_0 - AI_0}\right] \times 100 \tag{58.13}$$

In Eq. (58.13), AR_1 = activity of reactivated enzyme at t_{aging}, AR_0 = activity of reactivated enzyme at $t = 0$, AI_1 = activity of inhibited enzyme without reactivator at t_{aging}, and AI_0 = activity of inhibited enzyme without reactivator at $t = 0$. The slope (k_4) of the least-squares best-fit line from a plot of ln(100/% inhibition) versus t is determined using linear regression from the initial linear portion of the curve. From Eq. (58.12), it can be seen that the units of k_4 are t^{-1}, for example, min^{-1}, and that the aging half-life, $t_{1/2}$, the time required for 50% aging, can be determined from Eq. (58.14).

$$t_{1/2} = \ln 2/k_4 \approx 0.693/k_4 \tag{58.14}$$

The apparent half-life of aging depends upon the enzyme, inhibitor, pH, and temperature. However, NTE inhibited with most neuropathic agents exhibits aging half-lives on the order of several minutes, so that the 1–4-week delay between exposure and appearance of signs of OPIDN is not due to the time required for aging of phosphylated NTE (Jianmongkol et al., 1999; Johnson, 1982; Kropp and Richardson, 2007; Richardson, 1992).

58.5.5 Relative inhibitory potency

The neuropathic potential of an OP compound depends upon its ability to inhibit NTE (and presumably for the NTE–OP conjugate to undergo aging). However, an OP compound will not be able to inhibit NTE to a sufficient degree to produce OPIDN if its cholinergic toxicity is too high to permit survival of a neuropathic dose. Therefore, the neuropathic potential of an OP compound is a relative concept that depends upon the ability of the compound to inhibit NTE compared to its anti-AChE activity. Compounds can be screened in vitro for relative inhibitory potency (RIP) by measuring k_i or I_{50} values and calculating a ratio, as shown in Eq. (58.15).

$$\text{RIP} = \frac{k_i(\text{AChE})}{k_i(\text{NTE})} \tag{58.15}$$

Because of the reciprocal relationship between the k_i and I_{50} described by Eq. (58.8) above, the RIP for fixed-time I_{50} values (determined at the same fixed-time, t) is given by Eq. (58.16).

$$\text{RIP} = \frac{I_{50}(\text{NTE})}{I_{50}(\text{AChE})} \tag{58.16}$$

Thus, when the RIP > 1, the compound is a more potent inhibitor of AChE than NTE, and cholinergic toxicity would be expected to predominate over OPIDN. In fact, when the RIP > 1, the dose required to produce OPIDN tends to be greater than the median lethal dose (LD_{50}) for the compound (Kropp and Richardson, 2003; Richardson, 1992). Note that the RIP is simply a ratio of inhibitor potencies, and it could have been defined the other way around, with the k_i for NTE in the numerator. However, the convention used in this chapter defines the RIP as shown in Eqs. (58.15) and(58.16).

For example, although chlorpyrifos methyl oxon (CPMO), the active metabolite of the insecticide chlorpyrifos methyl, would seem to be a potent inhibitor of NTE, with $k_i = 5.82 \times 10^4\ \text{M}^{-1}\ \text{min}^{-1}$ and 20 min $I_{50} = 0.595\ \mu\text{M}$, it is a much more potent inhibitor of AChE, with a $k_i = 1.09 \times 10^7\ \text{M}^{-1}\ \text{min} - 1$ and 20 min $I_{50} = 3.18 \times 10^{-9}$ M (3.18 nM), so that the RIP for this compound is 187. This value is >> 1, indicating that OPIDN could not be produced unless the dose were >> LD_{50} and aggressive therapy for cholinergic toxicity were instituted (Kropp and Richardson, 2003). In contrast, the compound di-*n*-pentyl 2,2-dichlorovinyl phosphate (*n*-pentyl DCV) has an RIP of 0.03 and produces OPIDN at a dose of 2 mg/kg, which is much lower than the LD_{50} dose of 26 mg/kg (Richardson, 1992). OP inhibitors with extremely high selectivity for NTE have been synthesized, for example, ethyl *n*-octylphosphonofluoridate (EOPF) (Fig. 58.3) (Wu and Casida, 1995), which has 20-min I_{50} values for mouse brain NTE and AChE of 0.02 and 54 nM, respectively, yielding an RIP value of 3.7×10^{-4} (i.e., about 2700 times more potent for NTE than AChE) (Wu and Casida, 1996). Note that EOPF is a

chiral compound, and the reported I_{50} and RIP values are for the racemic mixture. Given that inhibitory potency can vary considerably between *R* and *S* forms of OP compounds (Wu and Casida, 1994), it would be of interest to carry out such measurements on resolved isomers.

Because RIP values can encompass several orders of magnitude above and below 1, it is convenient to visualize such data using log (RIP) values as shown in Fig. 58.8. It can readily be seen that higher positive numbers correspond to greater cholinergic propensity, as typified by the insecticide active metabolite, CPMO. Likewise, larger negative numbers correspond to greater neuropathic potential, as exemplified by the DN agent, EOPF. It is also interesting to see the steady progression from cholinergic to neuropathic tendency exhibited by the homologous series of dialkyl 2,2-dichlorovinyl phosphates (dichlorvos, DCV) compounds. In this and other series of alkyl OP inhibitors, increasing length of alkyl chains tends to produce decreasing potency against AChE and increasing potency against NTE until maximum selectivity is reached, after which potencies and selectivity decline (Richardson, 1992; Malygin et al., 2003). The highest potency for NTE appears to have been achieved with certain phosphonofluoridates, where the sum of alkyl and alkoxyl chain length is 12−16 atoms, including O and P; in this series, EOPF has the highest potency and selectivity for NTE (Wu and Casida, 1995, 1996).

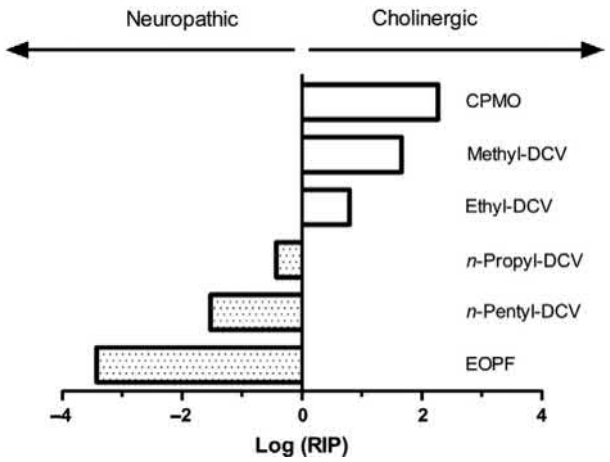

FIGURE 58.8 Spectrum of log values of relative inhibitory potency (RIP) = k_i(AChE)/k_i(NTE), where k_i = bimolecular rate constant of inhibition. As log(RIP) becomes more positive, cholinergic potential increases; as log(RIP) becomes more negative, delayed neuropathic potential increases. CPMO, chlorpyrifos methyl oxon. DCV derivatives refer to symmetrical dialkyl 2,2-dichlorovinyl phosphate (dichlorvos) compounds. EOPF, ethyl *n*-octylphosphonofluoridate. *Data from Kropp, T.J., Richardson, R.J., 2003. Relative inhibitory potencies of chlorpyrifos oxon, chlorpyrifos methyl oxon, and mipafox for acetylcholinesterase versus neuropathy target esterase. J. Toxicol. Environ. Health Part A 66, 1145−1157; Richardson, R.J., 1992. Interactions of organophosphorus compounds with neurotoxic esterase. In: J.E. Chambers, P.E. Levi (Eds.), Organophosphates: Chemistry, Fate, and Effects. Academic Press, San Diego, pp. 299−323; and Wu, S.Y., Casida, J.E., 1996. Subacute neurotoxicity induced in mice by potent organophosphorus neuropathy target esterase inhibitors. Toxicol. Appl. Pharmacol. 139, 195−202.*

Determinations of RIP carried out in vitro using preparations of AChE and NTE can only assess the neuropathic potential of OP compounds that are direct-acting inhibitors, that is, compounds that do not require metabolic activation. Such preparations would also not detect effects of detoxification that might occur in vivo. To account for the possibility of metabolic activation/deactivation, the system can be augmented by preincubating the compound of interest with xenobiotic metabolic enzymes (or chemical activators, e.g., bromine) before mixing with AChE or NTE preparations (Barber et al., 1999). The concept of the RIP can also be extended to assaying AChE and NTE ex vivo and calculating activity ratios after dosing laboratory animals with the compound of interest. Because the adult hen is the species of choice for assessing the neuropathic potential of OP compounds, this species could also be used for ex vivo RIP determinations (Richardson, 1992; Richardson et al., 1993). However, despite the fact that rats and mice are not as suitable as hens for studying the clinical and histopathological effects of DN agents, rodent species have AChE and NTE in their nervous systems and can therefore serve as sources of these enzymes for in vitro or ex vivo determinations of neuropathic potential (Novak and Padilla, 1986; Quistad et al., 2003; Wu and Casida, 1996; Makhaeva et al., 2014).

58.6 Biomarkers

58.6.1 Introduction

A widely quoted definition of "biomarker" is that it is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al., 2001). Although this definition was developed from the standpoint of pharmacology and therapeutics, it can easily be modified to apply to toxicology. In a toxicological setting, a biomarker would consist of an appropriate measure of a chemically induced pathological process, for example, a response to a toxicant that could be considered injurious or diagnostic of potential injury. Ideally, a toxicological biomarker would represent a biological response that would be pathognomonic of a specific agent or at least a selective indicator of the effects of a class of agents. Thus, a biomarker of a DN agent would involve more than measuring the mere presence of the compound; instead, it would assess a distinctive and preferably early feature of the pathogenesis of OPIDN. Because the earliest known molecular events

associated with OPIDN are the inhibition and aging of NTE, the appropriate biomarkers of DN agents would be measurements of these reactions, which could be done via determination of inhibition and aging using enzymology, or through identification of the resulting OP−NTE conjugates using mass spectrometry.

58.6.2 Enzymological measurements of neuropathy target esterase inhibition and aging

NTE activity in lymphocytes and platelets was discovered by Dudek and Richardson (1978, 1982), characterized in human populations (Bertoncin et al., 1985; Maroni and Bleecker, 1986), and used in animal (Makhaeva et al., 2003; Richardson and Dudek, 1983; Richardson et al., 1993; Schwab and Richardson, 1986) and human (Lotti et al., 1983, 1986; McConnell et al., 1999) studies to monitor exposures to potentially neuropathic OP compounds. NTE activity is not found in erythrocytes or in plasma or serum. These studies indicate that NTE in lymphocytes, platelets, or whole blood can be assayed and used as a biomarker of exposure to DN agents, particularly if the blood samples are taken within 24 h of acute exposures. Attempts to measure the aging of NTE in lymphocytes or platelets of exposed subjects have apparently not been done. However, given that aging of NTE inhibited by DN agents typically occurs within a half-life of a few minutes (Clothier and Johnson, 1979; Johnson, 1982), aging would be complete by the time that lymphocyte samples could be taken and assayed for activity. Nevertheless, as stated above, given the apparent requirement for aging as well as inhibition of NTE in OPIDN, to help rule out false positives arising from inhibition by type B (nonaging) NTE inhibitors (Fig. 58.5), aging studies ought to be undertaken in future work. As noted below, mass spectrometry can be used to identify the phosphyl adduct on the inhibited enzyme, thus differentiating between inhibited and aged forms.

In keeping with the concept of the RIP discussed in Section 58.5.5, inhibition of lymphocyte and/or platelet NTE and possibly erythrocyte LysoPC hydrolase should be used in conjunction with inhibition of erythrocyte AChE and plasma butyrylcholinesterase (BChE) to assess the likelihood that an exposure to OP compounds would produce cholinergic and/or DN effects. Erythrocyte AChE inhibition has long been used as a biomarker of exposure to conventional nerve agents or OP insecticides (Lotti, 1995; Wilson and Henderson, 1992). BChE can be sensitive to both conventional and DN agents, and its inhibition could thus serve as a general biomarker for OP agents (Jiang and Lockridge, 2013; Kropp and Richardson, 2007; Van der Schans et al., 2008).

58.6.3 Identification of neuropathy target esterase−organophosphorus conjugates using mass spectrometry

Mass spectrometry has proved to be an excellent tool for identifying the OP conjugates (adducts) of serine hydrolases such as AChE and BChE (Doorn et al., 2000, 2001b, 2003; Elhanany et al., 2001; Jennings et al., 2003; Jiang et al., 2013a,b; Kropp and Richardson, 2007; Li et al., 2007; Schopfer et al., 2014; Van der Schans et al., 2008). One approach is to reactivate inhibited esterases with fluoride and to detect the liberated OP fluoridate using gas chromatography−mass spectrometry; however, this technique cannot be used if the esterase conjugate has aged (Degenhardt et al., 2004; Solano et al., 2008). Because NTE inhibited with DN agents undergoes rapid aging (Johnson, 1982), it is important to be able to detect the aged adducts. Therefore, peptide mass mapping would be the current method of choice, whereby the active site peptide containing the phosphyl moiety on the active site serine is identified using mass spectrometry following digestion of the inactivated esterase with trypsin or other proteases (Doorn et al., 2003; Jiang et al., 2013a,b). Although some DN agents have extremely high potency and selectivity for NTE, in general there will be some degree of inhibitory overlap with other serine hydrolases (e.g., see Fig. 58.8). Accordingly, in addition to examining NTE, it would be useful to acquire mass spectrometry data on OP conjugates of other potential esterase targets, such as AChE and BChE, with particular attention being paid to the aged species.

The classical view of the aging reaction of OP-inhibited serine esterases, based on studies of soman-inhibited AChE, is that aging involves an SN1 reaction at a ligand carbon and transient formation of a carbocation that dissociates from the enzyme (Michel et al., 1967; Shafferman et al., 1996). The traditional model has been confirmed using mass spectrometry for BChE inhibited by a variety of phosphates and phosphonates and allowed to age in the presence of [18O]-H_2O (Li et al., 2007). Thus, it appears that cholinesterases inhibited by phosphates or phosphonates undergo aging via SN1 cleavage of an O−C bond rather than SN2 cleavage of a P−C bond.

However, it is important to realize that mechanisms of inhibition and aging can differ depending upon the esterase, inhibitor, and stereochemistry of the inhibitor. For example, the aging of AChE inhibited by ethyl *N*,*N*-dimethylphosphoramidocyanidate (tabun) was shown to proceed via hydrolytic P−N bond scission with loss of dimethylamine (Elhanany et al., 2001). However, BChE inhibited by tabun analogs was shown to age differently, depending on chirality (Jiang et al., 2013b). Thus, the *Sp* tabun analog aged via deamination, whereas the *Rp*

isomer aged by dealklation. In addition, the mechanism of inhibition of AChE, BChE, and cholesterol esterase by the resolved stereoisomers of isomalathion has been shown to differ between the (1*R*) and (1*S*) forms, and the mechanism of aging in each case appears to proceed via SN2 displacement of a secondary leaving group by water, involving cleavage of a P−S bond (Doorn et al., 2000, 2001a, 2001b, 2003). A subsequent study of BChE inhibited with racemic isomalathion confirmed that the major pathway for aging involved scission of the P−S bond, but relatively small peaks in the mass spectrum indicated that O−C and S−C cleavage could also occur as minor pathways (Li et al., 2007). Such studies are useful, in that they demonstrate the "signatures" of OP conjugates arising from different agents on particular esterases.

For NTE inhibited by phosphates or phosphonates, the aging mechanism has been thought to be consistent with an SN2 reaction at phosphorus (Johnson, 1982), although this hypothesis has not been rigorously tested. With respect to stereochemical influences on aging, limited studies indicate that both enantiomers of certain phosphonates inhibit NTE, but only one enantiomer undergoes aging (Johnson et al., 1985, 1986). As expected, the isomer that inhibits NTE and ages produces OPIDN. In contrast, the isomer that inhibits NTE without aging does not produce OPIDN. Furthermore, the nonaging isomer protects against neuropathy from subsequent administration of a neuropathic OP compound (Johnson and Read, 1987; Johnson et al., 1988). Some racemic phosphonates have been found to produce OPIDN at lower than expected threshold levels of inhibited/aged NTE (i.e., <70%), possibly because of potentiation or promotion of subclinical axonopathy by the nonaging enantiomer, presumably by the action of the nonaging enantiomer on one or more targets other than NTE that could be involved in repair or regeneration of neural lesions (Lotti, 1992).

Perhaps the most thoroughly investigated compound with respect to inhibition and aging of serine hydrolases is diisopropylphosphorofluoridate (DFP). Using a variety of techniques, this compound has been shown to inhibit AChE (Clothier and Johnson, 1979; Kropp and Richardson, 2006; Millard et al., 1999a, 1999b; Ordentlich et al., 1996, 1998), BChE (Kropp and Richardson, 2007; Li et al., 2007; Masson et al., 1997), NTE (Clothier and Johnson, 1979; Williams, 1983; Williams and Johnson, 1981), and NEST (Kropp et al., 2004) by organophosphorylation of the active site serine followed by aging to yield a monoisopropylphosphoryl adduct, as shown in Fig. 58.9. Esterase adducts have been directly demonstrated using mass spectrometry for AChE (Kropp and Richardson, 2006), BChE (Kropp and Richardson, 2007; Li et al., 2007), and NEST (Kropp et al., 2004), and in the case of BChE, the mechanism of aging has been unequivocally demonstrated to involve

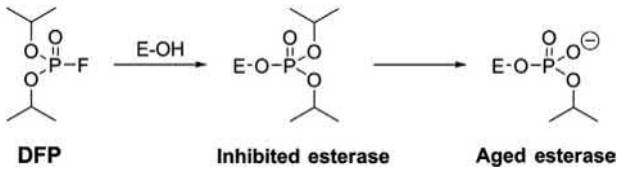

FIGURE 58.9 Inhibition and aging of serine esterases by diisopropylphosphorofluoridate (DFP). The active site serine is organophosphorylated in the inhibition step. Aging results in net loss of an isopropyl group to yield the monoisopropylphosphoryl esterase. This mode of inhibition and aging has been established for acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and neuropathy target esterase catalytic domain (NEST) (Kropp and Richardson, 2007).

scission of an O−C bond in an isopropyl group (Li et al., 2007). These results, taken together with those for soman-inhibited AChE (Michel et al., 1967) and BChE inactivated with a variety of phosphonates and phosphates (Li et al., 2007), indicate that it is reasonable to expect that aging of phosphonylated or phosphorylated serine esterases, including NTE and NEST, will proceed via O−C bond scission. This insight enables accurate prediction of the biomarker species to be sought in the mass spectra of serine esterases inhibited with phosphonates or phosphates.

Among the type A inhibitors of NTE (Fig. 58.5), the phosphoramidates are perhaps the most enigmatic to be studied to date. For example, mipafox, the phosphoramidate analog of DFP, is capable of producing suprathreshold inhibition (>70%) of neural NTE in vivo and causing OPIDN (Davis and Richardson, 1980). Therefore, mipafox-inhibited NTE was expected to undergo aging (Davis et al., 1985). Indeed, early studies showed that NTE inhibited by mipafox was intractable to reactivation, indicating the possibility of aging as a mechanism for yielding a nonreactivatable enzyme (Johnson, 1982). In the case of mipafox-inhibited NTE, however, nonreactivation was thought to be too rapid to measure by standard kinetic approaches (Johnson, 1982). Consequently, aging of mipafox-inhibited NTE had only been inferred and not directly demonstrated. Subsequently, Milatovic and Johnson (1993) showed that NTE inhibited by mipafox could be reactivated by prolonged treatment with KF at acidic pH. This surprising finding led to the conclusion that aging had not occurred after all. Instead, it was surmised that an unusual property of the bond between the phosphoramidate and the active site serine was responsible for the stability of the complex as well as the perturbation of NTE that leads to OPIDN.

It had been known for some time that esterases inhibited by mipafox are resistant to reactivation, but conventional aging could not be proved or disproved by classical techniques. It is also known that the phosphoramido hydrogen on *N*-alkylphosphoramidates is acidic and that its removal yields a resonance-stabilized anion

(Eto, 1974). Such a mechanism would explain the relative stability of phosphoramidated esterases in a manner that does not require loss of an alkylamino group to yield a negative charge on the adduct (Richardson, 1995). Moreover, reprotonation of the phosphoramide anion would generate a neutral species subject to attack by a nucleophile and account for the reactivation of mipafox-inhibited NTE at low pH. Under the acidic conditions usually employed for peptide mass spectrometry, the putative anion would be reprotonated. Thus, it can be hypothesized that the species attached to NTE after its inhibition by mipafox and allowing time for aging is the intact *N*,*N*′-diisopropylphosphorodiamido moiety. Such a result would indicate that the inhibited enzyme has not lost an isopropyl or isopropylamino group due to an aging process analogous to the net loss of an isopropyl group in the aging of DFP-inhibited NTE or NEST.

In contrast to the results for mipafox-inhibited NTE, early limited studies indicated that mipafox-inhibited BChE or AChE undergo irreversible aging, because the enzymes could be reactivated soon after inhibition, but not after 18 h (Milatovic and Johnson, 1993). The chemistry of phosphoramidates suggests that aging could involve hydrolytic loss of an alkylamino group. For example, acid-catalyzed P−N bond fission has been observed for certain *N*-alkyl phosphoramidates (Eto, 1974) and the aging of tabun-inhibited AChE has been shown to proceed via P−N bond scission with loss of dimethylamine (Elhanany et al., 2001).

Considerations of probable mechanisms of aging for mipafox-inhibited serine esterases suggest that mass spectrometry studies would support a deprotonation mechanism for NTE or NEST and hydrolytic P−N bond scission for AChE and BChE. These expectations were borne out for human recombinant NEST, which was used as a surrogate for NTE (Kropp et al., 2004), and BChE, respectively (Kropp and Richardson, 2007). However, mipafox-inhibited AChE gave the surprising result, confirmed by immunoprecipitation and mass spectrometry, of apparent loss of both isopropylamine groups to yield a simple phosphate adduct on the active site peptide (Kropp and Richardson, 2006). However, further mass spectrometric investigations of mipafox-inhibited human AChE that could not be reactivated found only the intact mipafox adduct on the active site peptide (Mangas et al., 2016). Moreover, mass spectrometric studies of the aging of tabun−BChE conjugates demonstrated that the *Rp* isomer of the tabun analog aged by dealkylation to yield a phosphyl adduct with a dimethylamine R-group attached (Jiang et al., 2013b). Furthermore, these investigators showed that P−N bonds are acid-labile, so that treatment of the *Rp*-tabun−BChE conjugate with acid liberated dimethylamine to yield a simple phosphate adduct on the BChE active site peptide. Thus, taking together the results of Jiang et al. (2013b) and Mangas et al. (2016), we now conclude that our report of "aging" of mipafox-inhibited AChE to yield a simple phosphate adduct was in fact an artifact of the acidic conditions used to prepare samples for mass spectrometry. Nevertheless, as pointed out by Jiang et al. (2013b), the acid-lability of P−N bonds can be used diagnostically in a stepwise mass spectrometric procedure to demonstrate the existence of acid-cleaveable phosphyl adducts on esterases.

Mass spectrometry has also been successfully employed to detect BChE products arising from reaction with 2-(2-methylphenoxy)-BDPO (CBDP), which has been implicated in aerotoxic syndrome (Carletti et al., 2011). Interestingly, the final aging product was a simple phosphoserine adduct. A subsequent study showed that sequential aging steps first yield an *o*-cresyl phosphoserine conjugate followed by formation of a simple phosphoserine adduct (Schopfer et al., 2014).

58.7 Biosensors

58.7.1 Nanostructured electrochemical biosensors to measure enzyme activity

Recently developed nanofabrication methods using layer-by-layer (LBL) self-assembly provide exciting new opportunities to design multilayered, functional, biosensor interfaces to measure enzyme activity. Since the LBL technique was first introduced (Decher and Hong, 1991), significant work has been done to fabricate polymer and organic thin films through alternating deposition of positively and negatively charged polyelectrolyte species on solid surfaces (Decher, 1997; Decher et al., 1992; Lvov et al., 1994; Ruths et al., 2000; Schmidt et al., 1999).

Polyelectrolyte layers typically have a thickness on the order of a nanometer, allowing ultrathin interfaces to be fabricated that have minimal mass-transfer resistance. LBL self-assembly using polyelectrolytes is thus well suited for biosensor development studies, because, by minimizing mass-transfer resistances, the intrinsic enzyme kinetics can be measured. LBL assemblies of polyelectrolytes have been used to develop nanostructured biosensor interfaces that encapsulate enzymes (Hassler et al., 2007; Kohli et al., 2006, 2007a; Sokolovskaya et al., 2005) and functional nanoparticles (Kohli et al., 2005, 2007b).

Electrochemical biosensors that measure the activity of a protein have been widely used to determine analyte concentrations, in both research and commercial applications (Prodromidis and Karayannis, 2002). Biosensors can detect protein activity either directly (Hassler and Worden, 2006) or indirectly through reaction coupling (Peteu et al., 1996, 1998). The indirect approach greatly

expands the range of enzyme activities that can be characterized using electrochemical biosensors. For example, reaction coupling has been used to measure NTE activity outside of the electrode, for example, in blood samples (Makhaeva et al., 2003, 2007, 2016a; Sigolaeva et al., 2010, 2013) or AChE, BChE, and NEST activity incorporated into the electrode (Kohli et al., 2007a, 2014). Differential inhibition of multiple esterases has been used to define the "esterase profile" of a compound—an index that can be used to predict the net biological effect of an esterase inhibitor (Makhaeva et al., 2016b).

58.7.2 Electrochemical biosensor arrays for high-throughput analysis

Microsystem technologies, particularly thin-film deposition of microelectrodes and formation of microfluidic channels, have been widely applied to biological analysis systems (Bergveld, 1996; Urban, 2000), such as DNA-processing chips (Raisi et al., 2004) and other lab-on-chip implementations (Kovacs, 2003; McFadden, 2002; Ziegler, 2000). Most of these devices rely on optical transduction mechanisms (Rabbany et al., 1994; Vo-Dinh et al., 2003) that often cannot quantitatively measure several important protein activities, such as redox reactions.

In contrast, enzymatic redox reactions are readily measured using versatile electrochemical techniques. Traditionally, electrochemical measurements are performed at the "beaker scale," but newer technologies allow such systems to be miniaturized for improved performance and high-throughput analysis. Complete three-electrode electrochemical cells, including thin-film Ag/AgCl reference electrodes, have been integrated on silicon surfaces (Yun et al., 2004). Thin-film reference electrode stability has been improved using Nafion or polyurethane coatings (Nolan et al., 1997) and complex micromachined structures (Suzuki, 2000; Suzuki et al., 1999). A remaining challenge is to adapt these new technologies to the construction of miniaturized electrochemical arrays for high-throughput protein activity characterization. Such biosensor array platforms must be compatible with the self-assembled biosensor interfaces, thus establishing requirements for electrode materials, geometries, surface smoothness, and overall array architecture (Huang et al., 2013).

58.7.3 Assembly of electrochemical biosensor interfaces for serine hydrolases

The natural reaction substrates and products of serine esterases are not electrochemically active. In order to transduce these esterase activities into an electrical signal, a two-enzyme reaction pathway can be assembled on a gold electrode. In the first reaction, the esterase of interest converts a phenyl ester (e.g., phenyl valerate) into phenol (Fig. 58.10). In the second reaction, tyrosinase converts phenol first to catechol, and then to *o*-quinone (Fig. 58.11), which can be electrochemically reduced back to catechol at an electrode, yielding a measurable current (Eq. 58.17) (Kohli et al., 2007a, 2010).

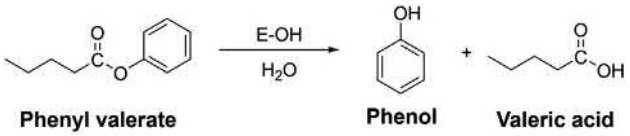

FIGURE 58.10 Reaction of a serine esterase with phenyl valerate to yield phenol and valeric acid (Kayyali et al., 1991).

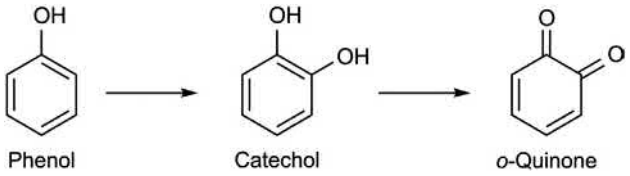

FIGURE 58.11 Reactions catalyzed by the two activities of tyrosinase. Phenol is first oxidized to catechol and then to *o*-quinone. *Reproduced with permission from Kohli, N., Srivastava, D., Sun, J., Richardson, R.J., et al., 2007a. Nanostructured biosensor for measuring neuropathy target esterase activity. Anal. Chem. 79, 5196–5203.*

$$o-\text{Quinone} + 2H^+ + 2e^- \rightarrow \text{Catechol} \qquad (58.17)$$

Tyrosinase is a copper-containing oxidase (Coche-Guerente et al., 2001; Forzani et al., 2000), which possesses the two different activities illustrated in Fig. 58.11. In the first step, referred to as the hydroxylase or cresolase activity, molecular oxygen is used to hydroxylate phenol to form catechol. In the second step, known as the catecholase activity, the enzyme oxidizes catechol to *o*-quinone, which is simultaneously oxidized by oxygen to its original form, with the production of water. The *o*-quinone is electrochemically active and can be reduced back to catechol, as illustrated in Eq. (58.17). Such electrodes have been used to determine esterase activities in blood samples (Sigolaeva et al., 2013).

By co-immobilizing tyrosinase with a serine esterase on a gold electrode, it is possible to establish a multistep reaction pathway that allows the activity of the esterase to be determined indirectly via measurement of *o*-quinone reduction at the electrode. The molecular architecture of a bi-enzyme sensor interface is shown schematically in Fig. 58.12 (Kohli et al., 2010).

In practice, the fabrication of these bi-enzyme biosensors is relatively straightforward. Gold electrodes are scrupulously cleaned in "Piranha solution" [(concentrated) H_2SO_4: 30% (w/w) H_2O_2, 7:3 (v/v)] and then dipped in a

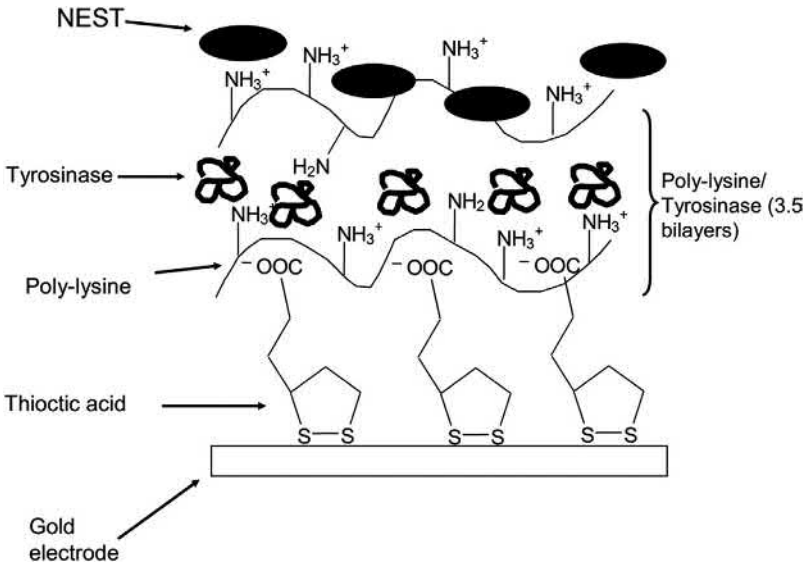

FIGURE 58.12 Molecular architecture of a bi-enzyme biosensor electrode containing tyrosinase and neuropathy target esterase catalytic domain (NEST). *Reproduced with permission from Kohli, N., Srivastava, D., Sun, J., Richardson, R.J., et al., 2007a. Nanostructured biosensor for measuring neuropathy target esterase activity. Anal. Chem. 79, 5196–5203.*

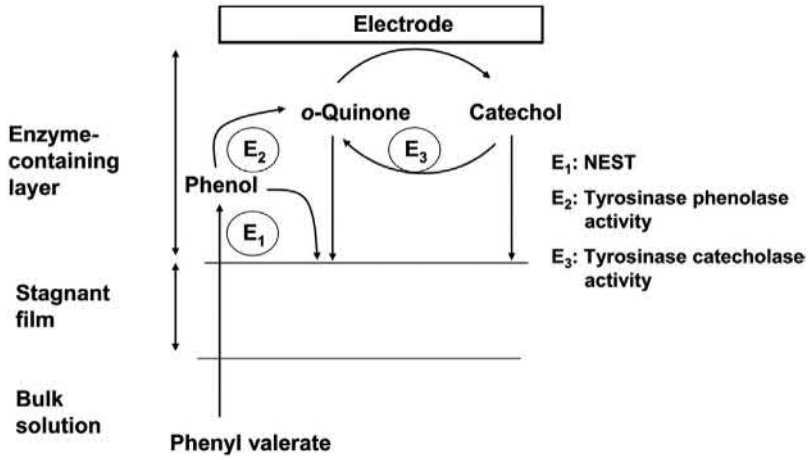

FIGURE 58.13 Schematic representation of the pathway that leads to the generation of current in the bi-enzyme biosensor containing tyrosinase and neuropathy target esterase catalytic domain (NEST). *Reproduced with permission from Kohli, N., Srivastava, D., Sun, J., Richardson, R.J., et al., 2007a. Nanostructured biosensor for measuring neuropathy target esterase activity. Anal. Chem. 79, 5196–5203.*

5 mM solution of thioctic acid in ethanol for 30 min. The electrodes are washed with ethanol, dried under nitrogen, and dipped in positively charged poly-L-lysine (PLL) solution for 45 min. The PLL solution is prepared by adding 12 mg of PLL in 50 mL of 20 mM phosphate buffer (pH 8.5). The electrodes are then rinsed with water and dipped in an aqueous solution of negatively charged tyrosinase (0.2 mg/mL) for 1 h. The last two steps are repeated 3.5 times to create 3.5 PLL-Tyr bilayers, with PLL being the topmost layer. The electrodes are washed with water and dipped in a 0.1 mg/mL solution of a serine esterase (e.g., AChE, BChE, or NEST) in 100 mM phosphate buffer, pH 7.0, for 1 h. The electrodes are then washed with water, dried under nitrogen, and dipped in phosphate buffer (0.1 M, pH 7.0) for testing. All steps are done at room temperature (approximately 22°C) (Kohli et al., 2007a).

58.7.4 Electrochemical measurements of serine esterase activity

The electrochemical properties of serine esterase-containing biosensor interfaces are readily characterized. Each biosensor is immersed in a stirred buffer solution and maintained at a potential of −100 mV (vs an Ag/AgCl reference electrode). For example, NEST activity was measured indirectly via the output current of the electrode for a variety of phenyl valerate concentrations. As shown in Fig. 58.13, the first step involves the diffusion of the ester (e.g., phenyl valerate) through a stagnant film from bulk solution to the enzyme layer. The NEST enzyme then hydrolyzes phenyl valerate to phenol, which is then oxidized to *o*-quinone by tyrosinase. Reduction of *o*-quinone at the surface of the electrode generates a current and regenerates catechol, which can then be

reoxidized by tyrosinase. This internal recycling between catechol and *o*-quinone provides a mechanism for signal amplification, thereby enhancing biosensor sensitivity (Kohli et al., 2007a).

The response of the biosensor to aliquots of ester can be measured at a variety of pH values and applied potentials. For a typical NEST biosensor, the highest signal-to-background ratio is obtained at pH 7.0 and a working electrode potential of −0.1 V relative to an Ag/AgCl reference electrode. At this potential, addition of phenyl valerate to a stirred aqueous solution triggers a rapid increase in the NEST biosensor signal, with a time constant of about 5 s. BChE can be substituted for NEST, with a similar current versus time response using phenyl valerate as substrate. For AChE, phenyl acetate is a better substrate than phenyl valerate. Current increases linearly with phenyl acetate concentration ($R_2 = 0.989$) in the range of 0.5−16 μM, reaching saturation at approximately 40 μM. Current versus time response and concentration calibration curves similar to those obtained for NEST and AChE can be obtained for BChE in the bi-enzyme electrode. Sensitivities of the electrodes obtained from the slopes of the initial linear portions of the concentration calibration curves were found to be 180, 25, and 87 $nA\mu M^{-1}\,cm^{-2}$ for AChE, BChE, and NEST, respectively, where AChE used phenyl acetate, and BChE and NEST used phenyl valerate as substrate. Control experiments were also done in which each of the substrates was delivered to a gold electrode containing only PLL-Tyr bilayers. The current sensitivities obtained in these control experiments were negligibly small—always less than 0.5 $nA\mu M^{-1}\,cm^{-2}$, indicating that the signal is mediated by the esterase activity (Kohli et al., 2007a).

The ability of the biosensors to detect chemical agents was confirmed by adding a known serine esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF), to the solution. The electrode response decreased in a concentration-dependent manner (Kohli et al., 2007a). Using a combination of biosensors containing different target enzymes, for example, AChE and NEST, it would be possible to obtain real-time differential data that could be used to distinguish exposures to conventional versus DN agents.

58.8 Concluding remarks and future directions

Fortunately, DN agents have not yet been used in warfare or terrorism. However, their possible use must be considered seriously, because it is straightforward to design and synthesize these compounds based on established methods in OP chemistry. Thus, a conventional insecticide or nerve agent can be reengineered to produce a DN agent. The use of DN agents would be catastrophic, because there are no accepted means of prophylaxis or treatment, and the effects are devastating and permanent. NTE is the presumptive target for initiation of OPIDN. Accordingly, lymphocyte NTE has been used as a biomarker of exposure, and the NTE catalytic domain (NEST) has been incorporated into a biosensor for detection of DN agents. However, the three-dimensional structure of NTE as well as its precise physiological function and role(s) in the pathogenesis of OPIDN and other neurodegenerative diseases remain to be elucidated. Therefore, further structural and mechanistic understanding is needed in order to improve upon existing biomarkers and biosensors and to develop preventive and therapeutic measures for DN agent exposures. Finally, because the early pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD), and motor neuron diseases (MNDs), including ALS, involve distal axonopathy (Salvadores et al., 2017), lessons learned from studies of NTE and OPIDN could be applied to developing interventions for these neurodegenerative diseases.

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Chapter 59

The cross-linking action of organophosphorus poisons; Implications for chronic neurotoxicity

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59.1 Introduction

Exposure to organophosphorus (OP) pesticides or nerve agents produces an acute toxic response, via a well understood mechanism (Masson and Nachon, 2017). Inhibition of acetylcholinesterase activity results in accumulation of acetylcholine and overstimulation of acetylcholine receptors. The consequence can be respiratory failure and death.

Another way in which OP can cause health issues has been identified in licensed pesticide applicators. These workers developed neurologic symptoms, though they had no history of acute toxicity from exposure to pesticides. It has been proposed that neurologic symptoms were caused by chronic low-dose exposure to OP pesticides (Kamel et al., 2007). Chronic low-dose OP exposure is associated with depression, anxiety, and suicide (Voorhees et al., 2017). Epidemiologic evidence also links low-dose OP exposure to increased risk of Parkinson's disease (Wang et al., 2014) and Alzheimer's disease (Yan et al., 2016). Studies in rodents confirm the relationship between chronic low-dose OP exposure and neurotoxicity (Moser et al., 2005; Hernandez et al., 2015; Voorhees et al., 2019).

A mechanism to explain neurotoxicity and dementia from long-term, low-dose OP exposure is needed. We propose a mechanism that involves a newly discovered function of OP, namely OP catalyzed cross-linking of proteins.

59.2 Chemical reactions of organophosphorus poisons

OP esters are highly reactive chemicals. The most studied reactivity of OP esters is covalent bond formation with the active site serine of the serine esterases including acetylcholinesterase, butyrylcholinesterase, and carboxylesterase. Initial OP modification of the active site serine of acetylcholinesterase and butyrylcholinesterase is followed by a reaction termed "aging" in which an alkyl group on the OP is displaced (Michel et al., 1967; Millard et al., 1999). This reaction results in essentially irreversible inhibition of the cholinesterases. In contrast, OP-inhibited human carboxylesterase-1 spontaneously regains activity with a half-life of 45 h after inhibition by sarin (Hemmert et al., 2010).

The acute toxicity of nerve agents and OP pesticides is due to inhibition of acetylcholinesterase activity at neuromuscular junctions. OP esters also make covalent bonds with the active site serine of serine proteases, including trypsin, chymotrypsin, and thrombin, though this has been documented only in vitro with purified proteins. The serine proteases are less sensitive to OP inhibition than acetylcholinesterase and butyrylcholinesterase, requiring higher concentrations of OP to achieve inhibition. The mechanism of OP adduct formation on serine has been known since 1954 (Schaffer et al., 1954).

A second action of OP was discovered in 2005 by analyzing OP-treated proteins using mass spectrometry (Peeples et al., 2005). It subsequently was found that OP make stable adducts on tyrosine and lysine in proteins that have no active site serine (Grigoryan et al., 2009; Lockridge and Schopfer, 2010). These studies with pure proteins were the foundation for the discovery of a new biomarker of OP exposure in human blood. It was found that humans who poisoned themselves by ingesting chlorpyrifos or dichlorvos have OP adducts on tyrosine 411 of serum albumin (Li et al., 2010, 2013; van der Schans et al., 2013). Studies

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in guinea pigs, rats, rabbits, and monkeys have shown that the nerve agents sarin, soman, cyclosarin, VX, and tabun also make stable adducts on tyrosine and lysine in albumin (Williams et al., 2007; Bao et al., 2012; Sun et al., 2017; Lee et al., 2018; Fu et al., 2019). Based on studies with pure proteins and cultured cells, it is expected that almost any protein can be modified on tyrosine and lysine by OP, though to date studies in humans and animals have identified only albumin OP adducts.

A third action of OP was discovered in 2014 when it was reported that the nerve agents VX, Russian VX, and Chinese VX catalyze the formation of internal cross-links in ubiquitin (Schmidt et al., 2014). Our laboratory confirmed the cross-linking action of OP, using chlorpyrifos oxon as the model OP. We found that treatment of pure proteins with 1.5 mM chlorpyrifos oxon resulted in internal cross-links as well as cross-links between proteins (Schopfer and Lockridge, 2018, 2019). The cross-links were between the epsilon amino group of lysine and the gamma carboxyl group of glutamic or the beta carboxyl of aspartic acid. Only specific residues were cross-linked. The degree of cross-linking for a particular pair ranged from about 1% to 10%.

59.3 Cross-linking mechanism

The mechanism by which OP esters cross-link proteins is illustrated in Fig. 59.1 with chlorpyrifos oxon as the OP. In the first step, chlorpyrifos oxon makes a covalent bond on the side chain of lysine in a protein. The products of the first step are a diethoxyphospho-lysine adduct in the protein and 3,5,6-trichloro-2-pyridinol. In the second step, the OP-modified lysine can react with the side-chain carboxyl group of a nearby glutamic acid. This yields an ε-aminolysine γ-glutamate isopeptide bond between two proteins and releases the diethoxyphosphate group from lysine. A similar reaction can occur with the side-chain carboxyl group of aspartic acid. These isopeptide bonds can link proteins to make high-molecular-weight fused protein aggregates, which can be seen on an SDS gel. Internal isopeptide cross-links within one protein are also found.

59.4 Mass spectrometry identifies cross-linked peptides

The isopeptide bond is resistant to proteolytic cleavage by trypsin and to fragmentation during electrospray mass spectrometry. This resistance makes it possible to identify cross-linked peptides by mass spectrometry. Dipeptides linked through an isopeptide bond are identified by their mass and fragmentation pattern. An example of mass spectrometry data for a cross-linked peptide is shown in Fig. 59.2.

A 1 mg/mL solution of purified porcine tubulin in pH 8.5 buffer was treated with 1.5 mM chlorpyrifos oxon for 7 days at 37°C (Schopfer and Lockridge, 2019). Control tubulin was treated under the same conditions but without added chlorpyrifos oxon. Excess chlorpyrifos oxon was removed by dialysis before the sample was reduced, carbamidomethylated, and digested with trypsin. The trypsin-digested sample was subjected to liquid chromatography-tandem mass spectrometry on the 6600 triple-TOF mass spectrometer (AB Sciex). Data were searched for cross-linked peptides using the free Batch-Tag program in Protein Prospector (University of California San Francisco Mass Spectrometry Facility http://prospector.ucsf.edu/prospector/mshome.htm). Protein Prospector reported MSMS spectra of potential cross-linked peptides.

It was essential to manually evaluate each MSMS spectrum to be sure the Protein Prospector assignment was a real cross-link. Our criteria for a cross-linked peptide are as follows. (1) The smallest peptide in the cross-

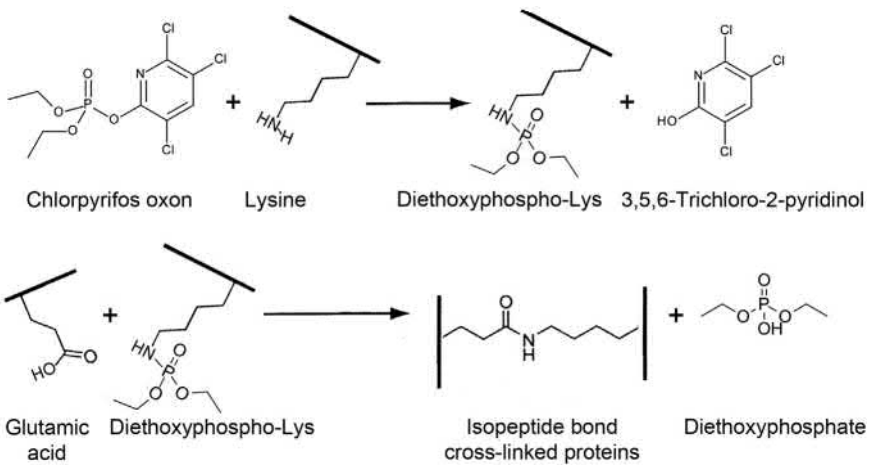

FIGURE 59.1 Chlorpyrifos oxon makes a covalent bond with the ε-amino group of lysine, thus activating the lysine for reaction with a nearby carboxyl group to yield cross-links between proteins and cross-links within a protein.

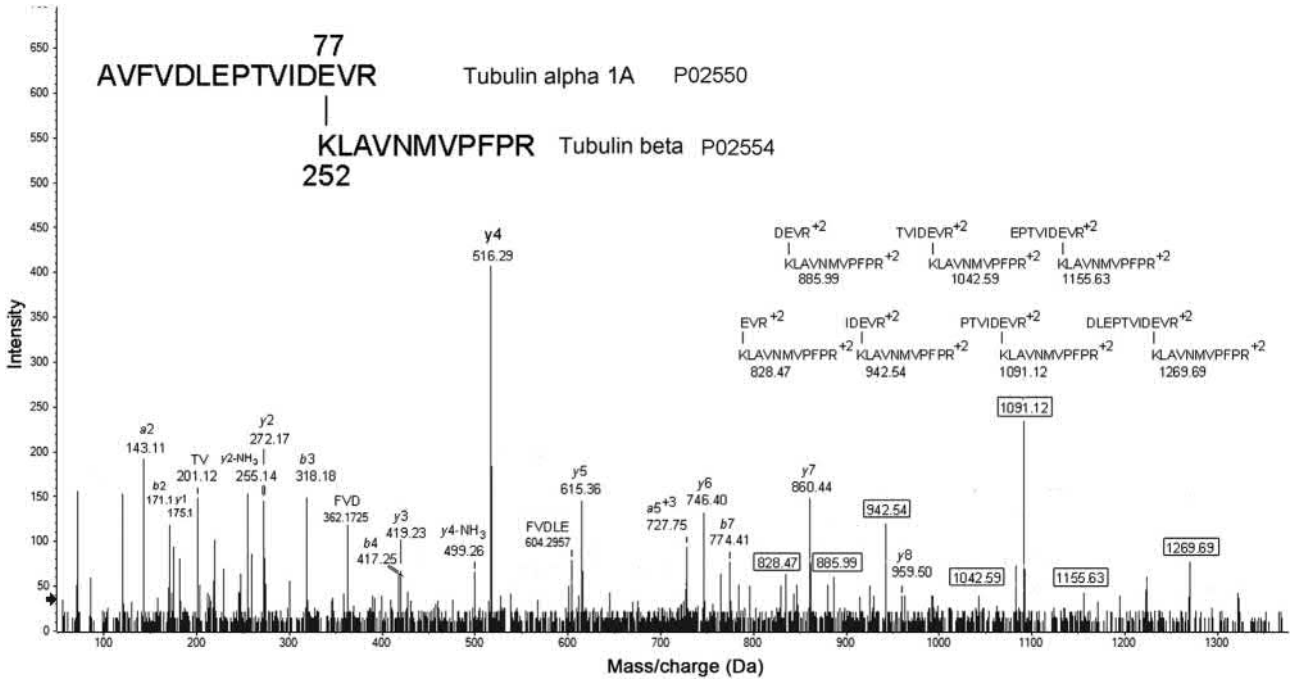

FIGURE 59.2 MSMS fragmentation spectrum of isopeptide cross-linked peptides produced by treatment of purified tubulin (*Sus scrofa*) with 1.5 mM chlorpyrifos oxon. The seven boxed masses are for fragment ions that support the cross-link between E77 of tubulin alpha 1A and K252 of tubulin beta. The structures of the doubly charged cross-linked ions are shown. A *b*-ion series for tubulin alpha 1A includes *b*2, *b*3, *b*4, and *b*7. A *y*-ion series for tubulin beta includes *y*1, *y*2, *y*3, *y*4, *y*5, *y*6, *y*7, and *y*8. The parent ion was positively charged with a monoisotopic mass/charge of 743.4085 for $[M + 4H]^{+4}$. The Uniprot accession number for tubulin alpha 1A is P02550 and for tubulin beta is P02554.

linked pair contains a minimum of four residues. (2) The mass of the parent ion is consistent with the mass of the two peptides minus 18 Da due to loss of a water molecule during the cross-linking reaction. (3) For cross-links between monomers of the same protein, the potential cross-linked peptide is not a contiguous linear sequence. (4) The primary amino acid sequences of both peptides are represented in the MSMS spectrum by a series of either b-ions or y-ions. (5) The MSMS spectrum of the cross-linked peptide candidate includes cross-linked fragments containing components from both peptide chains. (6) Control protein not treated with OP does not have the cross-linked peptide pair. The cross-linked peptide pair in Fig. 59.2 satisfies these criteria.

Cross-linked peptide pairs were also found when 0.1 mM chlorpyrifos oxon was used. Similar cross-links were found when 0.1 mM dichlorvos, chlorpyrifos (precursor of chlorpyrifos oxon), diazinon, diazoxon, and monocrotophos were used.

When 1.5 mM chlorpyrifos oxon was used, a portion of the lysine-containing peptide from the cross-linked pair was found to be modified on the lysine by diethoxyphosphate. But when 0.1 mM chlorpyrifos oxon was used, diethoxyphosphate labeling was not detected on the lysine peptide from the cross-linked pair even though cross-linking was seen. This suggests that the cross-linking reaction with activated diethoxyphosphate-labeled lysine is faster than the formation of diethoxyphosphate-lysine adducts when the chlorpyrifos oxon concentration is low.

59.5 The consequences of treating tubulin with chlorpyrifos oxon

Treatment of porcine tubulin, human albumin, mouse albumin, bovine casein, and human butyrylcholinesterase with 1.5 mM chlorpyrifos oxon yielded cross-links between lysine and glutamic acid, or lysine and aspartic acid. However bovine gelatin, bovine aprotinin, and chicken lysozyme formed no detectable chlorpyrifos oxon-induced cross-links. Tubulin was more susceptible to chlorpyrifos oxon-induced cross-linking than the other proteins, yielding 10 cross-linked peptide pairs in tubulin compared to two cross-linked peptide pairs in human albumin (Schopfer and Lockridge, 2019). Treatment with 1.5 mM chlorpyrifos oxon caused porcine tubulin to aggregate into nonreducible high-molecular-weight species as shown in Fig. 59.3.

Chlorpyrifos oxon perturbs GTP-induced polymerization of tubulin into microtubules, as shown in Fig. 59.4. Images were acquired by atomic force microscopy (Grigoryan and Lockridge, 2009). Bovine tubulin in pH 7.0 buffer was pretreated for 30 min with

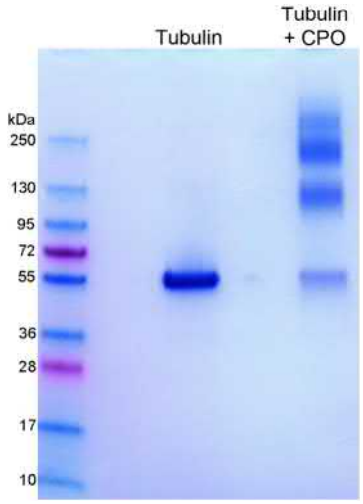

FIGURE 59.3 SDS polyacrylamide gel stained with Coomassie blue. Incubation of porcine tubulin with 1.5 mM chlorpyrifos oxon (CPO) for 7 days at pH 8.5 and 37°C, caused the 55 kDa tubulin to polymerize into nonreducible protein multimers.

0.005−0.10 mM chlorpyrifos oxon before GTP was added to initiate tubulin polymerization. In the absence of chlorpyrifos oxon, addition of 1 mM GTP polymerized tubulin into straight, well-structured, and individually separated microtubules (Fig. 59.4B). This can be contrasted with tubulin in the absence of GTP (Fig. 59.4A). Pretreatment with 0.005 mM chlorpyrifos oxon (Fig. 59.4C) caused a drastic reduction in the number and length of microtubules. Pretreatment with 0.010 mM chlorpyrifos oxon (Fig. 59.4D) increased the number of short microtubules. Pretreatment with 0.025 mM chlorpyrifos oxon (Fig. 59.4E) stimulated microtubule polymerization, increasing their density and clustering. Pretreatment with 0.050 mM chlorpyrifos oxon (Fig. 59.4F) caused the microtubules to clump. Pretreatment with 0.10 mM chlorpyrifos oxon (Fig. 59.4G) yielded short tubulin clusters. For comparison, the effect of a known microtubule polymerization inhibitor, colchicine, is shown in Fig. 59.4H. It was concluded that concentrations as low as 5 μM chlorpyrifos oxon interfered with microtubule formation.

Chlorpyrifos oxon disrupts the microtubule structure in vivo. We isolated microtubules from the brains of mice treated with nonlethal doses of chlorpyrifos oxon and examined the microtubules by atomic force microscopy (Jiang et al., 2010). We found that microtubules isolated from the brains of control mice were decorated with many proteins, but microtubules from chlorpyrifos oxon-treated mice had fewer associated proteins, suggesting disruption of microtubule function in vivo, as a consequence of exposure to chlorpyrifos oxon. Microtubules are polymers of tubulin dimers whose function is to maintain cell structure and provide tracks for transporting cell components between the cell nucleus and the axon. Disruption of the microtubule structure is consistent with our hypothesis (see below) that OP exposure causes cytoskeleton proteins (including tubulin) to cross-link, thus initiating the steps that lead to permanent loss of neuronal function.

59.6 Implications for neurotoxicity

A unifying hypothesis to explain neurodegenerative diseases including Alzheimer's, Parkinson's, Huntington's, and prion disease has been developed by Brady and coworkers (Brady and Morfini, 2017; Zamponi et al., 2017). The Brady hypothesis includes the following steps. (1) Aggregated proteins stimulate kinase activity in neurons. (2) The activated kinases phosphorylate motor proteins in excess. (3) The excessively phosphorylated motor proteins drop their cargo. (4) Loss of cargo deprives neurons of constituents that maintain neuron function. (5) Neurons slowly lose their connections to other neurons. (6) Clinical symptoms develop as a consequence of disruption of the neural network. Our finding that OP pesticides are cross-linking agents suggests a way to incorporate OP-induced neurotoxicity into the Brady hypothesis. Namely, OP toxicants promote formation of protein aggregates that initiate the process leading to clinical symptoms.

The published literature on the effects of OP treatment fits the Brady hypothesis in that OP exposure leads to aggregation of proteins in the neuron, to hyperphosphorylation of proteins involved in axonal transport, to impaired axonal transport, and to a decrease in neuronal connectivity. Specifically, studies in neuroblastoma cells, cell-free systems, and animals have shown that: (1) diazoxon and chlorpyrifos oxon induce protein aggregation in mouse neuroblastoma cells (Flaskos et al., 2011; Sachana et al., 2014). (2) OP induce hyperphosphorylation of neurofilaments (Gupta and Abou-Donia, 1995; Sindi et al., 2016), Tau protein (Gupta and Abou-Donia, 1999), calcium/cAMP-response element-binding protein (Schuh et al., 2002), tubulin and microtubule-associated protein 2 (Choudhary et al., 2001). (3) OP cause impaired axonal transport (Gupta et al., 1997; Gearhart et al., 2007; Hernandez et al., 2015; Naughton and Terry, 2018). (4) Mice treated with a subclinical dose of chlorpyrifos for 5 consecutive days had a 50% decrease in synaptic spine density 3 months after the last chlorpyrifos treatment (Speed et al., 2012), thereby reducing connectivity to other neurons.

59.7 Zero-length cross-links between lysine and glutamic acid or lysine and aspartic acid

We have found that treatment of pure proteins with chlorpyrifos oxon, dichlorvos, chlorpyrifos (precursor of

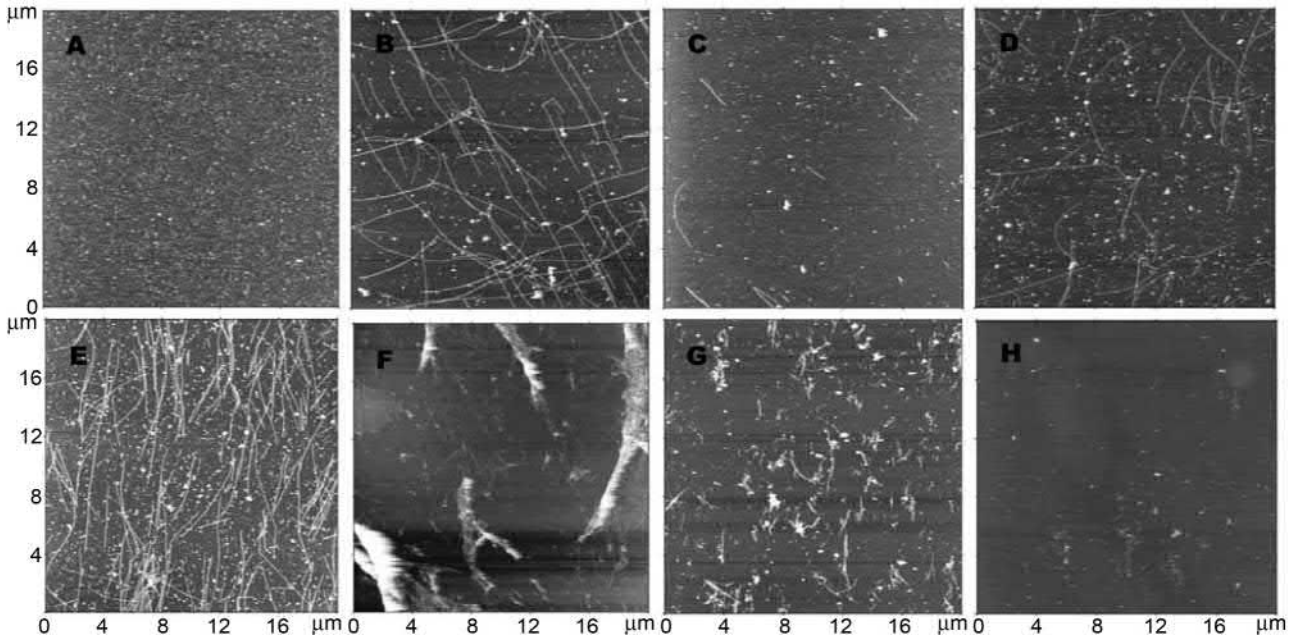

FIGURE 59.4 The effect of chlorpyrifos oxon on GTP-induced polymerization of tubulin, monitored by atomic force microscopy. (A) 0.1 mM bovine tubulin control, no GTP, no chlorpyrifos oxon; (B) 0.1 mM tubulin polymerized with 1 mM GTP, no chlorpyrifos oxon; (C) inhibition of GTP-promoted polymerization of tubulin by pretreatment with 0.005 mM chlorpyrifos oxon for 30 min before addition of GTP; (D) pretreated with 0.010 mM chlorpyrifos oxon before addition of GTP; (E) pretreated with 0.025 mM chlorpyrifos oxon before addition of GTP; (F) pretreated with 0.050 mM chlorpyrifos oxon before addition of GTP; (G) pretreated with 0.10 mM chlorpyrifos oxon before addition of GTP; (H) polymerization inhibited by 0.025 mM colchicine. The dimensions of each panel are 20×20 µm. *Reproduced from Grigoryan, H., Lockridge, O., 2009. Nanoimages show disruption of tubulin polymerization by chlorpyrifos oxon: implications for neurotoxicity. Toxicol. Appl. Pharmacol. 240, 143–148 with permission from Elsevier and Copyright Clearance Center.*

chlorpyrifos oxon), monocrotophos, diazinon, and diazoxon induces proteins to form cross-links between and within proteins. The residues in the cross-link are lysine and either glutamic or aspartic acid. The cross-link is zero length because atoms from the OP are absent in the cross-link. The mass of a cross-linked dipeptide is the sum of the masses for each peptide minus 18 Da for loss of a water molecule. The OP-activated lysine releases the OP during reaction with the carboxyl group to make the isopeptide bond (see Fig. 59.1).

The ability of OP to induce protein cross-linking is a novel finding. Schmidt et al. (2014) reported that VX made internal, zero-length, isopeptide cross-links within ubiquitin that resulted in cyclic structures. Schopfer et al. reported the cross-linking action of chlorpyrifos oxon on a variety of proteins yielding both internal, cyclic structures and protein–protein linkages (Schopfer and Lockridge, 2018; Schopfer and Lockridge, 2019). The only other chemical that is known to make zero-length cross-links is carbodiimide.

To date, no enzyme is known that catalyzes formation of an isopeptide bond between lysine and glutamic acid, or lysine and aspartic acid. Transglutaminase catalyzes cross-linking of the side chain of lysine to the side chain of glutamine to form a stable isopeptide bond (Murthy et al., 2009). Mammalian as well as bacterial transglutaminases have this capability (Bechtold et al., 2000). Mass spectrometry easily distinguishes between OP-induced (lysine to Glu or Asp) and transglutaminase-induced (lysine to Gln) cross-linked peptides. Future studies of human and animal tissues will be able to identify proteins cross-linked in vivo by exposure to OP.

59.8 Concluding remarks

In conclusion, we hypothesize that chronic neurotoxicity from low-dose OP exposure could be initiated by protein aggregates that develop as a consequence of OP-induced cross-linking activity.

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Chapter 60

Monitoring of blood cholinesterase activity in workers exposed to nerve agents

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60.1 Introduction

Determination of the activity of blood cholinesterases [red blood cell (RBC) AChE and plasma BChE] is currently the most important tool for confirmation of poisoning from a highly toxic organophosphate nerve agent (OPNA) for monitoring the recovery progress of an intoxicated person, and for forensic study purposes (Karalliedde et al., 2003; Pope et al., 2005; Bajgar, 2004, 2005; Eddleston et al., 2005; Pakravan et al., 2016). It is necessary to examine the whole picture of intoxication, that is, not only biochemical assays but also clinical signs, allowing more precise assessment of the prognosis for the intoxication. The evidence supporting AChE as the primary target of OPNA action has been confirmed and summarized by many authors (Bajgar, 1985, 1991; Taylor, 1985; Marrs, 1993; Marrs et al., 1996; Lotti, 2000). It includes the following observations: (1) symptoms of organophosphorus compound (OP) poisoning are similar to those of the AChE inhibitor physostigmine; (2) the in vivo LD_{50} value for a variety of OPs correlates well with the inhibition efficacy to AChE determined in vitro; and (3) cholinesterase reactivators (e.g., oxime reactivators), anticholinergics (e.g., atropine, benactyzine), and spontaneously reactivating AChE inhibitors (e.g., carbamates) can reduce OP toxicity. However, there is a variety of documented data showing that AChE inhibition is not the only important biochemical change in the body during intoxication. These data have described many other changes that accompany the development of intoxication and may contribute to OP toxicity. They have included changes to other enzymes (e.g., BChE, neurotransmitters, immune changes, anaphylactic reaction, behavior, etc.). The evidence includes the data indicating that prophylactic/therapeutic drugs might also have multiple sites of action (e.g., cholinergic or noncholinergic) similar to those observed during intoxication (Bardin and van Eeden, 1990; Bajgar, 1991, 1992; Cowan et al., 1996; Kassa, 1998; Soukup et al., 2010, 2013).

The first reaction of OPs is interaction with cholinesterases in the bloodstream (Bajgar, 1985, 1991; Benschop and de Jong, 2001) and then in the target tissues, the peripheral and central nervous systems (Bajgar, 1985, 1991; Bardin and van Eeden, 1990; Green, 1958, 1983; Marrs, 1993; Marrs et al., 1996). The delayed neurotoxic effect is due to something other than cholinesterase inhibition. The neuropathy target esterase (NTE) has been described as the main target site for this symptom, however, only some OPs are neurotoxic in that sense (Abou-Donia and Lapadula, 1990; Lotti, 1992; Johnson, 1990, 1992; Johnson and Glynn, 1995; Slotkin et al., 2008; Estevez et al., 2018).

The mechanism of AChE inhibition for all OPNAs is practically the same—it is enzyme inhibition via phosphorylation or phosphonylation of the esteratic (active) site of AChE. Monitoring the changes in cholinesterase activity and their development during intoxication is at present the best way for determining the severity of OP poisoning, as well as the reaction to antidotal therapy.

Both the toxicodynamics and toxicokinetics of OPNA can be explained by their biochemical characteristics of interacting with cholinesterases and other hydrolases in the

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organism. Inhibition of AChE and BChE in the blood is practically the first target for OPs according to the principle "first come, first served" (Benschop and de Jong, 2001).

AChE and BChE differ in their enzymatic properties and physiological functions (Massoulié et al., 1993; Darvesh et al., 2003; Thapa et al., 2017). Additionally, there are other cholinesterases, such as benzoylcholinesterase, propionylcholinesterase, etc. (Falugi et al., 2002; Hubbard et al., 2008). AChE splits neurotransmitter acetylcholine at the cholinergic synapses (synaptic clefts). It was also observed in the RBCs but its function is not fully known. It probably participates in cell-to-cell interactions (Assis et al., 2018). Similarly, the function of BuChE aktivity in plasma was only partially revealed, though there is evidence that BChE plays an important role in cholinergic neurotransmission and could be involved in other nervous system functions, including neurological diseases (e.g., Alzheimer's disease). BChE is also involved in nonspecific detoxification processes of ester-type local anesthetics and other compounds (Darvesh et al., 2003; Lockridge, 2015).

60.2 Determination of cholinesterases

Determination of cholinesterase activity is based on many principles. In general, an enzyme is added to the buffered or unbuffered mixture and a reaction begins once an appropriate substrate is added. Different parts of the reaction mixture are continually determined, that is, unhydrolyzed substrate or reaction products, either directly or indirectly (Augustinsson, 1971; Holmstedt, 1971; Witter, 1963). Conditions must be chosen very carefully (Dingova and Hrabovska, 2015; Reiner and Simeon-Rudolf, 2000).

According to the procedure and laboratory instrumentation, the most common methods of cholinesterase determination are as follows: electrometrical (e.g., Michel, 1949), titration (e.g., Nenner, 1970), manometric (e.g., Witter, 1963), colorimetric detection of the unhydrolyzed substrate (e.g., Hestrin, 1949), direct measurement of the pH change using an indicator (Dingova and Hrabovska, 2015; Winter, 1960), spectrophotometric (e.g., Siders et al., 1968; Voss and Sachsse, 1970; Worek et al., 1999a, b), fluorimetric (e.g., Sasaki, 1964; Kusu et al., 1990),

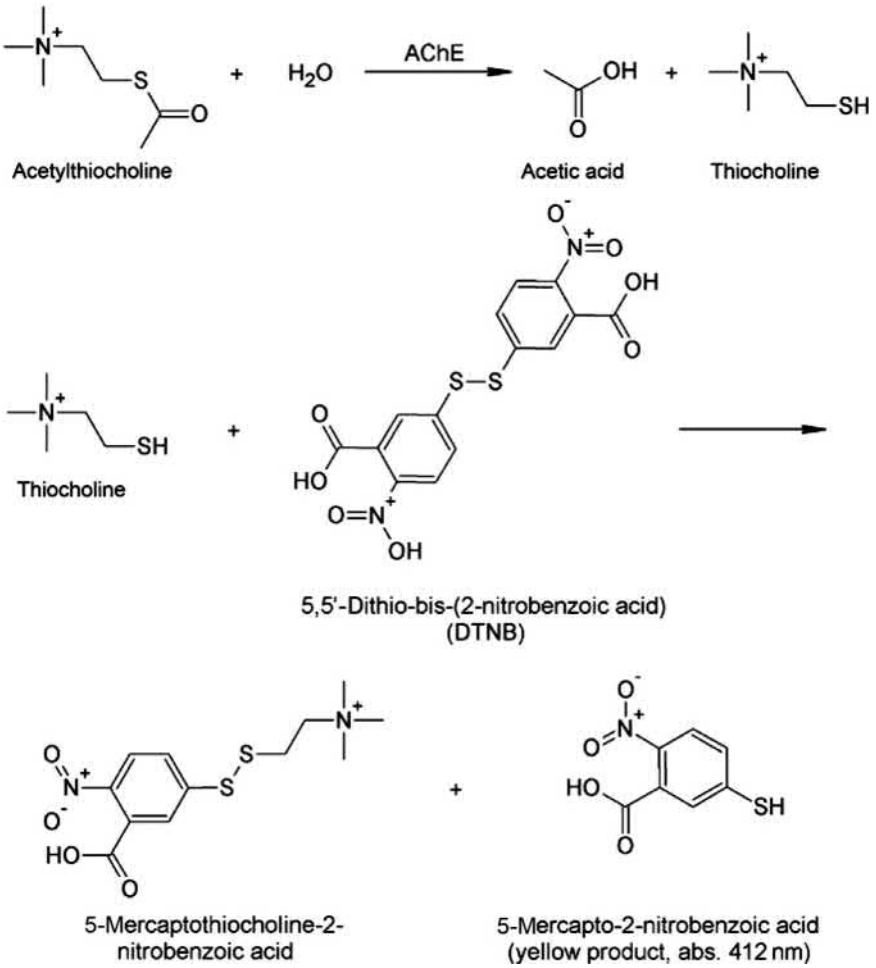

FIGURE 60.1 Acetylthiocholine is hydrolyzed by AChE/BChE to acetic acid and thiocholine, which forms yellow 5-mercapto-2-nitrobenzoic acid after reaction with Ellman's reagent.

radiometric (e.g., Israel and Lesbats, 1987), calorimetric (Konickova and Wadso, 1971), polarographic (Fiserova-Bergerova, 1969), enzymatic (Abernethy et al., 1986; Israel and Lesbats, 1987), and others, for example, near-infrared spectroscopy (Domjan et al., 1998). These methods are also suitable for the detection of cholinesterase inhibitors using biosensors (Brimijoin and Rakonczay, 1986; Ciriello et al., 2018; Cremisini et al., 1995; De Jong and Benschop, 1988; Miao et al., 2010; Walker et al., 2007) or immunochemical assay for detection of chemical warfare agents (Lenz et al., 1997a,b).

A very sensitive and commonly used method for cholinesterase determination was described by Ellman et al. (1961). This assay is based on hydrolysis of the thiocholine substrates acetyl- and butyrylthiocholine or others. When the substrate is cleaved, the relevant acid and thiocholine are released and thiocholine is detected by its SH-group using 5,5′-dithiobis-2 nitrobenzoic acid forming 5-mercapto-2-nitrobenzoic acid and determined spectrophotometrically at 412 nm (maximum absorbance). Slightly different wavelengths (e.g., 405 nm, 436 nm) may be used according to the specific instrument, to avoid interferences (Fig. 60.1).

Occasionally, the Ellman method is used with specific inhibitors in order to selectively inhibit the activity of either AChE or BChE and there are many modifications described in the literature. This method is in good correlation with other described methods. It is sufficiently specific and sensitive and it is used for different purposes in many laboratories around the world. Expression of AChE activity varies greatly, usually as μmoles of substrate hydrolyzed per min (time) per mL of material examined (e.g., plasma, serum) or per mg of tissue (wet, dry, mg of nitrogen, etc.). From these values, the expression of enzyme activity in international units (symbol: U) can be derived (quantity of enzyme catalyzing μmol of substrate per min at standard conditions). In the clinical laboratory, enzyme activity can also be expressed as katal (symbol: kat) per liter, that is, 1 mol of substrate hydrolyzed/s/liter or kg (kat/L or kat/kg), which is the hydrolysis of 1 mol of substrate hydrolyzed/s/liter or kg (mol/s/L or mol/s/kg). For conversion, 1 kat corresponds to 60,000,000 U or conversely 1 U corresponds to 0.000,000,016,666,666,7 kat. There are many publications addressing the review and modifications of cholinesterase determination. One of the last works written for improving Ellman's method (Ellman et al., 1961), including a description of the methods, is a paper published by Worek et al. (1999a).

60.3 Factors influencing the activity of cholinesterases

The influencing of BChE activity by gamma-irradiation, stress, gravidity, some neurological and psychiatric disorders, hormones, and medical drugs has been demonstrated (Brown et al., 1981; Bajgar, 1985, 1998; Darvesh, 2016). The elevation of BChE activity is not as common as an increase in children with nephritic syndrome has been observed. An elevated ratio of BChE/LDL cholesterol indicates an increase in the risk of cardiovascular diseases (Kutty, 1980; Navratil and Bajgar, 1987). The involvement of BChE in fat (cholesterol) metabolism has been studied and correlated (Han et al., 2019; van Lith et al., 1991; Van Lith and Beynen, 1993). The relationship between BChE activity and experimentally induced diabetes mellitus in rats is also mentioned (Annapurna et al., 1991). BChE activity in serum can be regarded as a biomarker for Parkinson's disease and related dementia (Dong et al., 2017).

In clinical biochemistry, BChE determination in plasma or serum is more frequently used than that of AChE in RBCs. Except for intoxication with OPs or carbamates, a BChE decrease indicates either a decrease in enzyme synthesis or a decrease in the number of production cells in the liver (Masopust, 1983). A special case of diminished BChE activity is the hereditary presence of atypical variants of BChE (Brown et al., 1981; Whittaker, 1980). There are many other factors influencing BChE activity and the diagnostic importance of diminished BChE activity is important for the following states: congenital deficiency, liver damage, acute infection, inflammation, chronic malnutrition, metastasis (especially liver), myocardial infarction, dermatomyositis, intoxication with carbon disulfide or mercury, and obstructive jaundice (Kutty, 1980; Molphy and Ratthus, 1964; Bardin and van Eeden, 1990; Bajgar, 1991).

Determination of AChE activity is not generally performed in clinical laboratories. A decrease in RBC AChE activity in pernicious anemia has been demonstrated; diminished erythrocyte AChE activity is typical for paroxysmal nocturnal hemoglobinemia and ABO incompatibility (Rakonczay, 1988). AChE activity in the erythrocyte membrane can be considered an indicator of membrane integrity. Increased AChE activity in rectal biopsy is of great diagnostic significance in Hirschsprung's disease, especially in the presence of its atypical molecular form (Bajgar and Hak, 1979; Rakonczay, 1988; Saldanha, 2017). There are other papers demonstrating increased AChE activity in the amniotic fluid during pathologic development of the neural tube (Bonham and Attack, 1983). AChE activity in the erythrocytes and cerebrospinal fluid is also diminished in some endogenous depressions and other psychiatric disorders and is probably also connected with behaviors related to stress situations. However, the results presented are not quite clear at present (for a review see, e.g., Bajgar, 1985; Rakonczay, 1988; Bohnen et al., 2007; Martucciello, 2008; Saldanha, 2017).

60.4 Diagnosis of organophosphorus compound poisoning

The determination of cholinesterases in the blood is the basic method for diagnosis and therapy monitoring for OP poisoning, though some doubts exists, preferring the clinical signs of poisoning as a leading tool for diagnosis and monitoring (Bardin and van Eeden, 1990). The determination of AChE and BChE activity in whole blood is possible. The decrease in these activities is a good marker but diagnostic validity is limited in that some factors causing a decrease in blood cholinesterases are present. This information is important, along with the anamnestic data (exposure to OP). The determination of red blood cell AChE or plasma BChE is more informative. There are some discussions regarding whether AChE or BChE activity is more important for diagnosis. In general, AChE activity in the red blood cell can be considered more important for diagnosis of nerve agent poisoning than plasma BChE activity. However, there are some discussions dealing with the validity of BChE determination. This enzyme has been described as a poor marker of OP poisoning and it has been proposed to exclude its analysis from recommended biochemical procedures (Molphy and Ratthus, 1964; Wyckoff et al., 1968; Bardin et al., 1987a,b). The temporal profile of BChE was studied in a cohort of 25 OP-poisoned patients to examine their relationship to the development of intermediate syndrome. The study suggests that BChE reflects the clinical course of poisoning and that intermediate syndrome may be associated with persistent BChE inhibition (Khan et al., 2001). Israeli authors also described a direct correlation between the degree of BChE inhibition levels and the severity of intoxication with OP (Weissmann-Brenner et al., 2002). According to Aygun et al. (2002), in the acute phase of OP poisoning, a low level of AChE supports the diagnosis of OP poisoning but it does not show a significant relationship to the severity of poisoning. Cander et al. (2011) confirmed the validity of AChE activity for the quick diagnosis of poisoning, however its efficiency at predicting outcome in patients with OP intoxication has not been established. The preference of AChE determination has been demonstrated by Worek et al. (1999b)—BChE activity determination for diagnosis and therapeutic monitoring provides no reliable information on AChE status. This is in agreement with the author's experimental results (Bajgar, 1998). In some cases, plasma BChE activity is a good marker for diagnosis of OP pesticide poisoning. It is necessary to exclude diminishing BChE activity caused by other reasons. In all cases, simple cholinesterase determination gives information about the decrease in enzyme activity without specification of the factor causing this phenomenon. A more detailed specification is possible using special methods not available in all clinical laboratories.

For occupational medicine purposes, the determination of cholinesterases in the blood of workers with OP is obligatory. A decrease of activity below 70% of normal values is an indicator that the worker should not come into contact with OPs. However, the normal values varied within the laboratories depending on the method/specific conditions of determination.

For practical purposes (individual and interindividual variation), determination of individual norm activity is recommended (this approach is better than calculating the decrease from an average value), as well as a separate determination of both cholinesterases. Enzyme activity in whole human blood corresponds to about 10% of BChE and 90% of AChE. This is different from rats where this activity ratio is 29% of BChE and 71% of AChE (Bajgar, 1972). Erythrocyte AChE activity seems to be more useful for these purposes than BChE activity in the plasma.

There are other biological materials available for special purposes—amniotic fluid, cerebrospinal fluid, and bioptic materials. From these samples, tissue obtained via rectal biopsy is used most frequently (diagnosis of Hirschsprung's disease). Elevated AChE activity in the rectal tissue/homogenate (detected histochemically/biochemically) is a good diagnostic marker indicating a need for surgical treatment of Hirschsprung disease and a criterion for the diagnosis and management of obstipation (Kobayashi et al., 2002). The presence of an unusual AChE band after electrophoretic separation supports the diagnosis (Bajgar and Hak, 1979; Rakonczay, 1988). The same (either AChE elevation or the presence of a new electrophoretic AChE form) in the amniotic fluid can be applied for the diagnosis of malformation of the neural tube development during pregnancy (Bonham and Attack, 1983). AChE activity in the cerebrospinal fluid is also changed in some pathological states; however, this information has little diagnostic validity and can be considered a complementary analysis (Koponen and Riekkinen, 1991).

In OP poisoning, it is necessary to check vital functions (cardiac, ventilation) and other clinical signs and, per symptoms, to apply different biochemical analyses and treatment. Diagnostic criteria are mostly based on patient history and respiratory symptoms (Bardin et al., 1987a,b; Bardin and van Eeden, 1990). Serum electrolytes (especially potassium), blood urea nitrogen (BUN), and creatinine are necessary to assess the degree of volume depletion by the secretory losses. Arterial blood gas, blood pH, glucose, lactate, and pyruvate allow assessment of the degree of hypoxia/hypercapnia/acidosis in the presence of respiratory distress. The neurological examination includes recording of the muscle action potential of M. abductor digiti minimi after stimulation of N. ulnaris. A quantitative correlation could be shown between red blood cell AChE activity and paraoxon concentration in

the plasma. In these cases, muscle function was severely disturbed when red blood cell AChE activity was inhibited more than 90% (Thiermann et al., 2002).

Direct determination of the toxic agent (OPNA) in the circulating system is also possible. However, the parent compound will circulate intact for a short period of time and detection will not be possible after a few hours of exposure. Metabolites circulate for a longer time period and are mostly excreted in the urine. A metabolite of sarin (*O*-isopropyl methylphosphonic acid) could be traced in urine and plasma from victims after the Tokyo subway sarin terrorist attack (Noort et al., 1998, 2002). For some OP pesticides (parathion, paraoxon), detection of *p*-nitrophenol in the urine is an indicator of exposure (Bajgar, 1985). However, the retrospectivity of these methods is limited. Detection using an immunoassay of nerve agents is now in progress. The antibodies against soman may have the appropriate specificity and affinity for immunodiagnosis of soman exposure (Lenz et al., 1997a,b; Miller and Lenz, 2001). Antibodies directed against certain NA were also considered for treatment of NA intoxication (Grognet et al., 1993).

The methods for determination of blood cholinesterase inhibition (AChE and BChE) do not allow identification of the OP and do not provide reliable evidence for exposure at inhibition levels less than 10%−20%. Moreover, they are less suitable for retrospective detection of exposure due to de novo synthesis of enzymes. Recently, a method was developed which is based on reactivation of phosphylated cholinesterase and carboxylesterase (CaE) by fluoride ions. Treatment of the inhibited enzyme with fluoride ions can inverse the inhibition reaction, yielding a restored enzyme and a phosphofluoridate, which is subsequently isolated and quantified by gas chromatography and phosphorus-specific or mass spectrometric detection (De Jong and van Dijk, 1984; Polhuis et al., 1997; Polhuijs et al., 1997). Human (and monkey) plasma does not contain CaE but its BChE concentration is relatively high [70−80 nM (Myers, 1952; De Bisschop et al., 1987)], much higher than the concentration of AChE in blood [c.3 nM (Heath, 1961)]. The plasma of laboratory animals, such as rats and guinea pigs, contains considerable concentrations of CaE, in addition to cholinesterases. This method allows partial identification of the OP, whereas the lifetime of the phosphylated esterase (and consequently the retrospectivity of the method) is only limited by spontaneous reactivation, in vivo sequestration, and aging. The rate of the latter process (aging) depends on the structure of the phosphyl moiety bound to the enzyme and on the type of esterase. Phosphylated CaEs generally do not age. Based on this method for retrospective detection of exposure to OP, the exposure of victims in the Tokyo incident to an OP, probably sarin, could be established from analysis of their blood samples (Fidder et al., 2002; Polhuis et al., 1997; Polhuijs et al., 1997). Fluoride-induced reactivation of OP-inhibited AChE is a reliable and retrospective method to establish OP exposure. It is limited to compounds that could be regenerated with fluoride ions. A novel and general procedure for diagnosis of exposure to OP, which surpasses the limitations of the fluoride reactivation method, has been described (Van der Schans et al., 2002). This method is based on the rapid isolation of BChE from the plasma by affinity chromatography and digestion with pepsin, followed by liquid chromatography and the mass spectrometric analysis of phosphylated nonapeptides, which result after the pepsin digestion of inhibited BChE. The method can be applied for the detection of exposures to various OP pesticides and NA (including soman). This approach is very valuable and represents a new field for the improvement of diagnosis with NA and OP. A comprehensive review of the methods for retrospective detection of exposure to toxic scheduled chemicals has been published by Noort et al. (2001, 2002). The same principle, digestion of enzyme and subsequent analysis using liquid chromatography and tandem mass spectrometry, was successfully used for determination of NA exposure using RBCs. Before the determination, human RBC AChE was immunopurified in order to obtain a sufficient amount of inhibited enzyme for analysis (Dafferner et al., 2017).

As was mentioned previously, a decrease in cholinesterase activity is indicative (after the exclusion of other factors) of an exposure to OPNA or other cholinesterase inhibitors. This simple determination does not allow for decisions regarding antidotal therapy (especially the repeated administration of reactivators) and it has low prognostic validity. Therefore, a new test of the reactivation has been described (Bajgar, 1991). The principle of the reactivation test is double determination of the enzyme, the first without and the second with the presence of a reactivator in the sample. The choice of reactivator is dependent on the availability of the oxime, however, in principle it is necessary to have in these parallel samples the same concentrations of reagents. The concentration of the reactivator (usually trimedoxime, but other oximes such as obidoxime, pralidoxime, or HI-6 are also possible) must not be higher than the oxime concentration which causes the oximolysis (hydrolysis) of the substrate (acetyl- or butyrylthiocholine), that is, the oxime concentration is lower than 10^{-3} M because higher concentrations of oximes cause artificial hydrolysis of the substrate (Patocka et al., 1973; Worek et al., 2012).

OP poisoning is very complex and there are many biochemical changes to be determined. The assessment of sensitivity and specificity was rather subjective, however, it is clear that the following parameters are the most sensitive: (1) cholinesterase determination, (2) possible determination of OP metabolites in the blood, and (3) possible determination of the phosphonyl moiety on the target enzyme. In intoxications where convulsions occurred, tension of the blood gases is also a good marker; however,

these changes are not very specific. The same approach can be applied to lactate. This is not surprising because of the existence of convulsions, including convulsions of the diaphragm (and thus disturbed ventilation, low oxygen saturation, and an increase in acid metabolites). It should also be mentioned that the validity of these parameters changes during intoxication. The changes in transaminases, LDH and γ-GT, indicating liver damage can be caused by solvents used in commercially available OP insecticides. A low validity in the number of RBCs or leukocytes is also indicated. As for CS and TAT—stress markers—it is clear that OP intoxication represents a stress situation. In this scenario, an increase in ALT can also be considered a stress marker and not indicative of liver damage.

Determination of AChE or BChE molecular forms can be interesting and useful for improvement of the diagnosis of OP poisoning. It was demonstrated that these forms are inhibited in different manners—some forms are resistant (low molecular weight), and others are very sensitive (high molecular weight). When total AChE activity is determined, the value obtained is a "mean of the activities of these forms." The changes in the cyclic nucleotides are interesting but not very valid for blood. They were determined during animal experiments with toxic OC and are of more interest in connection with the nervous system. Esterases and AP, generally hydrolases, are sensitive but the inhibition potency of different OPs is variable. For nerve agents, these hydrolases are not especially valid, but for some OP insecticides (malathion) they are sometimes more sensitive than cholinesterases. In conclusion, diagnosis of OP poisoning represents a serious problem. The development of new specific methods (fluoride reactivation, tandem MS analysis of enzymatic digestion of AChE or BChE) is of high importance for more precise diagnosis of OPNA poisoning. An extensive review by Noort et al. (2002) addressing biomonitoring of exposure to chemical warfare agents (not only nerve agents) is strongly recommended. From a practical point of view in the clinical laboratory, it is necessary to monitor basic physiological functions, cholinesterases, and other biochemical parameters not only for diagnostic purposes but also preferably for the regulation of treatment.

60.5 Monitoring of blood cholinesterase activity in workers exposed to nerve agents

60.5.1 Introduction

For occupational medicine purposes, the determination of cholinesterases in the blood of workers exposed to OPs is obligatory. However, the normal values vary within the laboratories depending on the method of analysis.

Systematic monitoring of workers exposed to nerve agents was performed at the University of Defense, Department of Toxicology, during 1962−63. However, results presented expand over 40 years (1964−2004). Due to the large amount of data, the results are limited to RBC AChE only, though plasma BChE activity was also determined.

60.5.2 Methods for determination

Modified Hestrin's method (Hestrin, 1949) was used initially. This method is based on colorimetric detection of unhydrolyzed substrate, acetylcholine. Acetylcholine reacts with hydroxylamine quantitatively in alkaline solution after a reaction with $FeCl_3$. The activity was determined in RBC hemolysate distilled water (1:20). The method was modified and activity was expressed as μmoles of hydrolyzed acetylcholine/min/mL.

In 1964, a method described by Winter (1960) using an Auto Analyzer (Technicon, United States) system was evaluated. This method is based on determination of acetic acid released from the hydrolyzed substrate (acetylcholine) and detected with phenol red. AChE activity in RBC hemolysate (1:10, distilled water) was determined (pH 7.6, phosphate buffer, 37°C). The activity was expressed as μmol of acetic acid/10 min.

From 1972 to 1975, Ellman's method (Ellman et al., 1961) modified for Auto Analyzer was used. Enzyme activity was expressed as ncat/L. The same method was used for enzyme determination using a kinetic method and Vitatron (Eefde, Holland, 976-90) and Uvicon 952 (Kontron Instruments, Switzerland instrumentation, 1991−2004).

60.5.3 Correlation among methods

To achieve comparable results (unification of the activity, i.e., one scale), all activities were recalculated to nkat/liter (nkat/L). Recalculation of enzyme activity was performed as follows:

U/L = 16.6667 nkat/L. However, when using different substrates (esters of choline and thiocholine), correction was made according to correlation analysis ($y = 2.0188x + 2.2458$ and $r_{xy} = 0.9998$). Moreover, this correlation was demonstrated previously including not only Ellman's and Winter's methods (Ellman et al., 1961; Winter, 1960), but also a potentiometric method.

60.5.4 Subjects

International inspectors: 28 males (two determinations, 2000 and 2002), 7 selected (more determinations), age unknown.

Students: 21 medical students, 10 female, 11 male, age 19–20 years.
Workers in the Department of Toxicology: 18 males, from these, 9 were selected for long-term study; and 23 females, from these 13 were selected for long-term study. Last determination was performed at age 60 (female) and 65 (male).
Age at the beginning of study: 24–40 years (male), 19–48 years (female). All determinations were performed from 1964 to 2004.

60.5.5 Statistical analysis

Due to the large number of data, only RBC AChE results are shown. A simple survey began with division of AChE activities (female and male). The next step was analysis of individual results and trends. Some attempts were made to detect a decrease of AChE activity in case of potential exposure to nerve agents. The results are not fully evaluated and may be considered preliminary.

60.5.6 Results and discussion

The results with Hestrin's method (Hestrin, 1949) are documented as an average activity of RBC AChE. Normal AChE activity was determined to be 104.9 ± 8.5 nkat/L and coefficient of variation was 27.1%. This method was relatively difficult and data are shown for information only. The results of AChE activities for all persons are shown in Fig. 60.2.

Selected results of RBC AChE activity determination for all results are shown in Table 60.1.

The correlation of activities in hemolysate and whole blood is shown in Fig. 60.3.

In general, there were some tendencies to decrease cholinesterase activity, especially RBC AChE activity. Some rare examples of suspect intoxications were observed in the cholinesterase monitoring as well as an RBC AChE decrease in workers during their work with high concentrations of nerve agents (e.g., inhalation exposure experiments) (Fig. 60.4).

In a practical way, the best picture of cholinesterase was achieved using individual norms for RBC AChE. This appears to also be a sensitive parameter for monitoring cholinesterase changes in exposed workers, followed by sensitivity for whole blood hemolysate. Plasma BChE activity is not as specific or sensitive a parameter. A 30% decrease of individual normals seems to be critical for further consideration. In conclusion, cholinesterase

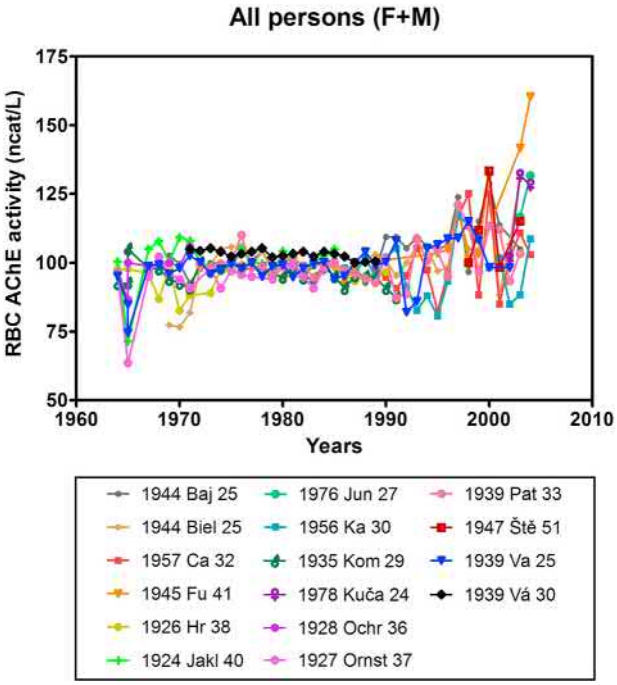

FIGURE 60.2 AChE activities of all monitored persons.

TABLE 60.1 Selected results of RBC AChE activity determination (nkat/L). Asterisks indicate significant differences between reference and measured activities, $*p < 0.05$.

Dept. of Toxicology	All	*M*	*F*
(18 + 23)	101.2 ± 7.2	102.2 ± 6.9	100.2 ± 8.4
Selected (9 + 13)	98.0 ± 8.2	99.7 ± 6.3	93.9 ± 7.8
Inspectors (0 + 28)	–	110.7 ± 9.4	–
(2000 + 2002)	–	112.4 ± 14.7	–
Students (11 + 10)	110.2 ± 9.5	115.1 ± 9.4	105.3 ± 9.5
Suspect intoxications (3 + 4)	90.9 ± 4.8*	94.3 ± 6.9*	86.4 ± 2.3*

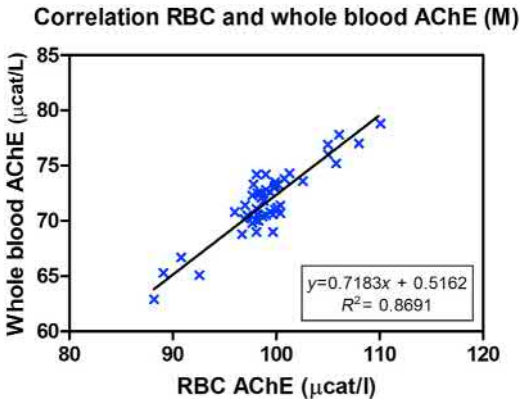

FIGURE 60.3 Correlation between RBC and whole blood AChE.

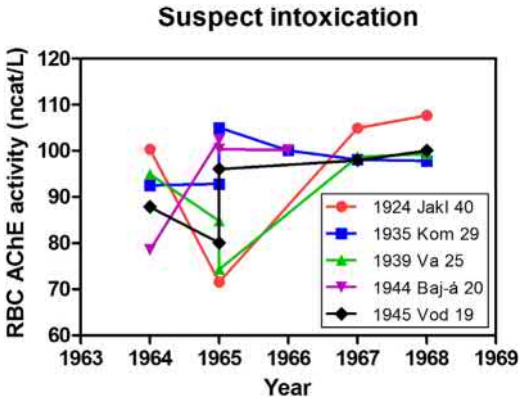

FIGURE 60.4 Suspect nerve agent intoxication.

determination in blood (especially RBC AChE) is a good parameter for assessing and monitoring workers who are exposed to highly toxic nerve agents.

Variation of RBC AChE is relatively high. Therefore, individual levels were considered more accurate, and the 30% decrease from individual norms was selected to be the limit of exclusion of workers from active work with nerve agents. Further decrease can be considered a significant diagnostic marker for suspected intoxication. According to the decrease, classification of steps of intoxication can be assumed: a decrease by 30%–50% indicates mild poisoning, further inhibition (50%–70%) medium, and severe (70%–90%) intoxication. This decrease is in good agreement with results on human and animals (Bajgar, 1992). An observed higher AChE activity for males was not significant. This coincides with other results (Augun et al., 2002). Similar results were obtained from other authors showing no significant differences in RBC AChE activity between males and females (Rumenjak, 1998) with a tendency toward increased activity with age (not significant). In general, the values of RBC AChE activity are quite comparable with results from other authors (Augun et al., 2002; Rumenjak, 1998; Zhou et al., 2003).

60.6 Concluding remarks

1. Sensitivity of blood cholinesterases as a diagnostic parameter for monitoring of workers exposed to nerve agents was demonstrated.
2. Sensitivity to detect some changes in incidents of nerve agent exposure decreases as follows: RBC AChE > whole blood cholinesterases > plasma BChE.
3. Normal enzyme activity detected with different methods correlates well.
4. RBC AChE or whole blood cholinesterase can be considered a good marker for monitoring nerve agent exposure.

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Chapter 61

Potential agents that can cause contamination of animal feedingstuff and terror

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61.1 Introduction

Foodstuffs (intended for human consumption) and feedingstuff (any single or multiple materials, including supplements, whether processed, semiprocessed, or raw, which is intended to be fed to food-producing animals) are vulnerable to terrorist attacks and forensic actions (Kosal and Anderson, 2004). A terrorist attack is intended to cause political instability and/or loss of a sense of security by inflicting injury, death, and economic loss to civilian populations (WHO, 2002). The organizations and individuals that commit acts of terrorism generally have multiple objectives (Spitzmuller and Park, 2018). In general, their motives focus on inflicting challenges to economic, social, and political stability, and intimidation of the target population. The interactions between food and terrorism are complex (Adelaja et al., 2018). This chapter is on a specific aspect of agroterrorism and has the theme of one medicine (one health) concept, wherein human health is interconnected to animal feedingstuffs because harmful chemicals in animal feedingstuff can become harmful residues in animal-sourced foodstuffs (Fig. 61.1) (Buttke, 2011). Most authors, in their discussions on agroterrorism, focus on the weaponization of infectious agents (WHO, 2002; Buttke, 2011; Keremidis et al., 2013). However, chemical and physical agents, used as weapons and forensic activities, are equally important in targeting feedingstuff (Neher, 1999; Kosal and Anderson, 2004). Finished feeds and supplements can contain cereal grains, food processing byproducts, rendered fats, etc. sourced from differing nations, and contamination of animal feedingstuff can result in multinational contamination of animal source foodstuffs (Fig. 61.1). Using chemical or physical agents, the terrorists can kill food-producing animals and/or create a food safety issue (Neher, 1999; Kosal and Anderson, 2004). Potentially harmful residues to human health can occur with and without the exposed food-producing animals showing signs of ill health. These residues can result in regulatory actions that remove these products from the market and can result in costly and highly debated disposal procedures (Shah, 1978).

Illegal residues of chemicals and/or radioisotopes in animal-source foods has both one-health and economic consequences. Terrorists contaminating feedingstuff can result in illegal residues in foodstuffs and have a large financial impact (van Larebeke et al., 2001; Lascano Alcoser et al., 2011; Hoogenboom et al., 2015; Hens et al., 2016). A specific case study showed that one incident of contamination of feed-grade animal fat with liquid transformer dielectric resulted in feedingstuff being contaminated with polychlorinated biphenyls (PCBs), chlorinated dibenzofurans, and polychlorinated dibenzodioxins/furans (PCDFs/PCDDs). This incident cost the Belgian government about €465 million, exclusive of any impact on human health (Buzby and Chandran, 2003). Essential defenses against agroterrorism and agrocrime can be expensive and come with opportunity costs because countermeasures require diversion of resources that could otherwise be used for enhancing agricultural enterprises. Emergency preparedness must include infrastructure and professional personnel for all events and hazards inclusive of terrorism and acts of agrocrime against livestock, poultry, and companion animals and the animal-to-human food web (Saliki, 2000). This infrastructure also serves in

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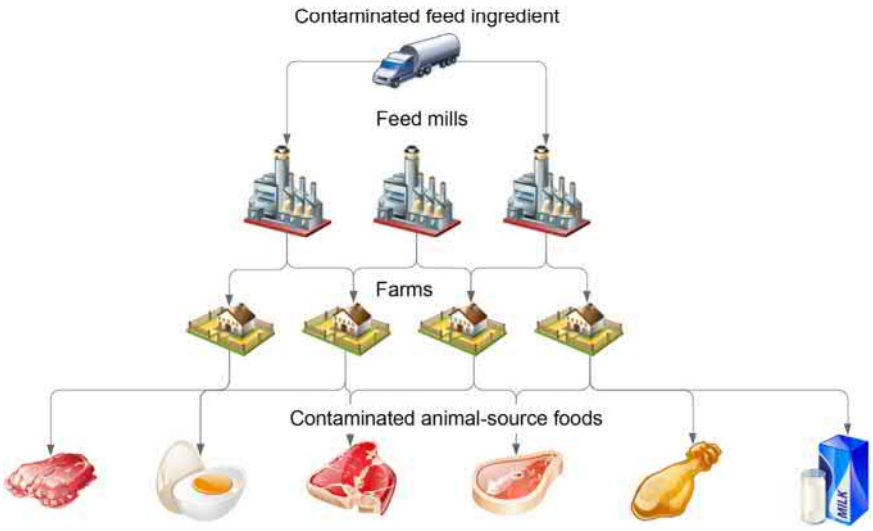

FIGURE 61.1 This diagram shows the potential for distribution amplification of chemically contaminated feedingstuff and the potential for large-scale human exposure to harmful and illegal chemicals. *Credit: B.E. Coppock.*

the diagnosis and prevention of diseases in domestic and wild animals. Timely decision making is essential in determining if the etiology of diseases in livestock is chemical−physical agents or infectious agents (Neher, 1999; Kosal and Anderson, 2004; Malisch, 2017; EU, 2018). The toxicologic focus of an investigation often lags until laboratory evidence shows the likelihood of infectious etiology is unlikely. Laboratory information that provides timely holistic scope and etiology of the incident is essential for timely decision making and food safety. The required infrastructure for comprehensive emergency preparedness includes full-service government-subsidized diagnostic laboratories for animals. Acts committed by terrorists and criminal perpetrators can overwhelm laboratory, regulatory, public health, and other resources (WHO, 2002; Kosal and Anderson, 2004). Well-rehearsed multiple-hazard protocols to deal holistically with an attack on food-animal production and the food supply chain are essential for emergency preparedness and a coordinated prompt response inclusive of food safety.

The FAO recognizes the safety of feedingstuff as being required to ensure animal health and food safety (FAO, 2015). The challenges in the feedingstuff industry are meeting the demand while ensuring feed safety. There is an increase in the diversity of byproducts from food and biofuel manufacturing that are used as ingredients in animal feedingstuff and the feed manufacturing industry has worldwide sourcing of these materials (FAO, 2008). Chemical and physical hazards in feedingstuff can have a negative impact on human health and impose a large expense on public and private sectors. Hazards can be introduced into feedingstuff with the source materials or through carryover as well as introduced during handling, storage, and transportation (FAO-IFF, 2010). The presence of the offending substance(s) can result from accidental or deliberate contamination by forensic or terrorism activities. Handling and manufacturing facilities provide opportunities for point source act-of-terror-linked adulteration of feedingstuff that results in wide distribution of chemical and physical agents (Fig. 61.1) (Kosal and Anderson, 2004). Depending on the feedingstuff, specific industries and livestock sectors can be targeted with significant economic losses. The product produced at the farm could be a target with large economic consequences (Wein and Liu, 2005). Terrorist attacks can also indirectly contaminate feedingstuff with chemical and physical agents resulting in collateral damage to the human food chain. For example, radioactive substances from a dirty bomb can contaminate forages, cereals, and other feedingstuff, and emissions from a fire can be deposited over a large area of agricultural land. Remobilization of radioisotopes from the Chernobyl exclusion zone by wildfire is considered a risk (Evangeliou et al., 2014). Smoke from a fire in a building containing PVC plastic contaminated growing corn with PCDDs/DFs. The corn was harvested as silage and was subsequently found to contain PCDDAs/DFs. A study on the contaminated silage showed the PCDDs/DFs were transferred from silage to milk (Hoogenboom et al., 2015). Persistent organic pollutants (POPs) have potential for weaponization for use by terrorists to adulterate animal feeds with the sole purpose of creating widespread harmful and illegal residues in animal-source foodstuffs with the potential for significant economic consequences including to human health. The practice of feeding animal fat back to animals can concentrate POPs in the human food web. Many of the POPs are bioconcentrated in food-producing animals and a large feedingstuff contamination incident has been shown to have multigenerational effects in humans (Blanck et al., 2000; Small et al., 2011; Terrell et al., 2015;

Walker et al., 2019). Companion animals can be exposed through chemically contaminated animal products being incorporated into pet foods (Brown et al., 2007). Attacks on companion animal feedingstuff resulting in illness and deaths in pets could have considerable emotional impacts on animal owners. The melamine−cyanuric acid adulteration of protein imported into the United States for formulating pet foods caused ill-health and deaths (Puschner et al., 2007). This incident was a criminal activity to increase profits by falsely increasing assay results for protein by the addition of melamine−cyanuric acid and was not related to terrorism.

61.1.1 Agricultural food ecosystem and terror

A brief review of the agri-food ecosystem is important in the discussion of terrorist-linked toxicology of animal feedingstuff. Generally, the activities and technologies in agriculture are maximized for human control and economic gain. All aspects in feedingstuff production, including supplements, drugs, and chemicals, can be targets for terrorists. Harmful agents and organisms can be directly or indirectly added to feedingstuff. Additions of regulatory agency-approved feed additives and drugs that are off-label for a particular livestock species or off-label in terms of excessive amounts added to the feedingstuff can cause animal deaths and/or illegal residues in edible animal products (Doonan et al., 1989; Kosal and Anderson, 2004; Baynes et al., 2016). Some drugs and feed additives have approved label usage in one country and are illegal to use in another (Centner et al., 2014). Thus, adulterants could be added forensically or added by terrorists to disrupt commerce by causing a food safety incident. The vulnerability of animal feeds can be illustrated by the reported large-scale toxicological incidents inclusive of chemical residues in edible animal products. Feed contamination incidents have important economic and human health consequences (Carter, 1976; Lok and Powell, 2000; Kosal and Anderson, 2004; Malisch, 2017; Walker et al., 2019). There is likely an underreporting of occurrences of feedingstuff-linked animal intoxications, with fragmentary governmental infrastructure to collect, process, and use veterinary toxicoepidemiological data (Guitart et al., 2010).

In agriculture, the margin between safe and unsafe practices is exceptionally close. Time- and weather-sensitive farming activities include seeding, watering, fertilizing, weed, and other pest control, harvesting inclusive of methods, preservation-storage, and shipping. All aspects of the system require that chemical contamination of feedingstuff does not occur, or the chemicals used in production remain within government-specified maximum allowable limits by following withdrawal times, etc. The chemicals used in the agroecosystem are essentially unprotected and are vulnerable to contamination attacks by terrorists. Terrorist attacks may not be directly focused on the agroecosystem but can indirectly narrow the margins between safe and unsafe agricultural practices and have huge impacts on feedingstuff and foodstuffs. For example, terrorists can disrupt the distribution of electrical and fossil fuel energy, availability of water, and also target critical industries, logistics, and infrastructure serving the agricultural sectors. Disruption of harvesting and preservation of feedingstuff increases the risk for proliferation of toxigenic fungi and mycotoxin production. Disruption of water availability can stress growing plants and increase the susceptibility of a crop to plant pathogens, including field fungi that produce mycotoxins. The agroecosystem food web also includes manufacturing of feedingstuff and foodstuffs. Food-producing animals in the agroecosystem are vulnerable to mycotoxins and persistent chemicals that are bioconcentrated and these can be relayed to humans via edible animal products (Malisch, 2017). Animal byproducts containing chemical residues can be "recycled" and used as ingredients in feedingstuff.

61.2 Mycotoxins and toxigenic fungi

61.2.1 Background

There are alleged uses of mycotoxins as chemical weapons (Tucker, 2001; Haig, 1982). Mycotoxins and mycotoxigenic fungi could be used for terrorist attacks on both feedingstuff and foodstuffs (Klassen-Fischer, 2006; Rai and Varma, 2010). In addition to generally low consideration of mycotoxins as the etiology of disease, the clinical presentations caused by mycotoxins are not unique and can be misdiagnosed (Paterson, 2006). Thus, a delay in the identification of mycotoxins as the causal agent(s) could occur over a considerable period of time. Delay in recognition can increase the number of animals and humans intoxicated. A large-scale human and animal exposure to mycotoxins occurred during World War II because the agroecosystem was disrupted by the war (Joffe, 1978). There are endemic exposures to mycotoxins in Africa and other parts of the world because of drought, economic hardships, wars, and agricultural practices. Mycotoxin contamination of foodstuffs and feedingstuff can be "collateral damage" from terrorism and war because of disruptions to agricultural practices, manpower, logistics, disruption of water availability, and restricted supply of energy resources. Toxigenic fungi are considered to have ubiquitous distribution. The triggers for mycotoxin production preharvest are growth-stressed plants with reduced resistance to fungal infections, and temperature and moisture levels in stored foods and feedingstuff (Coppock and Jacobsen, 2009). Mycotoxin

production can occur in the field, in wet conditions, and during transport and storage. Foodstuffs, considered unfit for human consumption, can be diverted to animal feedingstuff and cause mycotoxicosis in domestic animals, including pets. In addition to adverse effects on human and animal health, mycotoxin contamination can cause substantial economic hardship because of trade restrictions and bias.

61.2.2 Applications of biotechnology

It should be assumed that the effective use of mycotoxins as terrorist weapons can be increased with the use of biotechnology. Development of fungi for bioterrorism has been reported to share technologies with the military development of fungi for use as weapons (Paterson, 2006). It is possible for aggressive strains of toxigenic fungi to be isolated or developed that produce mycotoxins at lower temperatures and moisture levels than would be considered the usual limits for a particular fungal species (Miller, 2016; Moretti and Susca, 2017). Also, fungal varieties can be genetically modified (GM) in a manner that would enhance toxin production by removing the environmentally activated "restrictors" of toxin production. The adaptation of mycotoxigenic fungi and mycotoxins for terrorist activities likely follows the science used in fungal genetic research and biotechnology.

61.2.3 Fungal biocontrol agents

Fungal biocontrol agents (FBCAs) could be used in terrorist activities. Some FBCAs have been developed using bioselection and others using GM technologies (Paterson, 2006). A number of FBCAs produce mycotoxins (toxigenic fungi) and have been used to control unwanted vegetation. FBCAs are candidates, for example, *Fusarium oxysporum*, can be used to reduce the biomass produced of certain plants that are the source of illicit drugs (Charudattan et al., 2011). The same technologies can be used to target crops used as feedingstuff in livestock and poultry production and in the production of plant fibers.

61.2.4 Economic losses from the use of fungi and mycotoxins as weapons

The use of toxigenic fungi and mycotoxins for bioterrorism can have significant economic consequences inclusive of the impact on human and animal health. Increasing the environmental prevalence of aggressive strains of toxigenic fungi could increase the estimated world impact of mycotoxins from $2.5 billion to a substantially higher number. The estimated losses may be underestimated because projected annual loss in the United States from aflatoxin contamination is estimated at a maximum of $1.7 billion/year without including the economic impact on human and animal health (Mitchell et al., 2016). International commodity trading of dry cereal grains could be used as a method of disseminating the spores of GM aggressive toxigenic strains of a particular fungus. Once released into the environment, the GM fungi would likely be impossible to control. Many plant fungal pathogens are also producers of mycotoxins (Logrieco et al., 2002). The use of plant fungal pathogens as weapons would most likely increase the probability of crops being contaminated with mycotoxins. In national and international trade, feedingstuff and foodstuffs contaminated with mycotoxins can influence consumer/buyer preferences and trade, and have devastating economic consequences, especially in developing countries (Unnevehr, 2002).

61.2.5 Terrorism using mycotoxin-contaminated feedingstuff

Incidences of mycotoxicoses in livestock caused by imported feedingstuff that are not linked to terrorist activities have been reported from the use of mycotoxin-contaminated byproducts in formulating ruminant, horse, poultry, and swine feedingstuff (Loosmore and Markson, 1961; Wilson et al., 1990). As a terrorist tactic, mycotoxin-contaminated feedingstuff could be purchased at a bargain price and resold. For example, mycotoxin-contaminated feedingstuff ingredients could be exported and used to formulate animal feeds in multiple countries importing the ingredient. The backlash of such an event could have major economic consequences on the exporting country. Strains of toxigenic fungi that produce mycotoxins that are not commonly included in mycotoxin screens during import testing could be used with huge economic consequences (Gruber-Dorninger et al., 2017). There are incidents not linked to terrorism, where pet foods have been contaminated with aflatoxins and subsequently caused illness and deaths of companion animals (Arnot et al., 2012; Wouters et al., 2013).

61.2.6 Residues in edible tissues

Food-producing animals ingesting mycotoxin-contaminated feedingstuff can have residues in edible animal products (Coppock and Dziwenka, 2019; Coppock et al., 2018). Incidents of mycotoxin-contaminated feedingstuff being fed to food-producing animals and subsequent transfer of mycotoxins and their toxic metabolites into foodstuffs are a public health concern (Volkel et al., 2011). Residues of some mycotoxins in feedingstuff and animal-source foods are regulated in most countries. The concern for residues of mycotoxins in edible animal products has primarily been focused on regulated residues of aflatoxins in dairy

products. Terrorist-linked contamination of feedingstuff with mycotoxins would have an economic impact on trade in all dairy and other animal-source foods.

61.3 Microbial toxins

61.3.1 Botulism toxin

61.3.1.1 Background

The highly toxic botulism neurotoxins (BoNTs) produced by *Clostridium botulinum* are potential terrorist weapons via addition to foodstuffs and feedingstuff and are given priority as a choice by belligerents for weaponization (Woudstra et al., 2013). The saprophytic anaerobe, *C. botulinum*, is a ubiquitous Gram-positive spore-forming organism and BoNTs can easily be produced at a low cost. Botulism generally occurs after preformed BoNTs are consumed in foodstuff and feedingstuff. Botulism can also occur by *C. botulinum* growing in vivo in the gut or in anaerobic wounds causing toxicoinfection (examples are infant botulism, visceral botulism in domestic animals, and wound botulism) (Anniballi et al., 2013; Mariano et al., 2019). Potential routes of exposure to BoNTs and *C. botulinum* spores are oral, inhalation, and dermal (wound botulism). BoNTs can be added directly to foodstuffs and feedingstuff. The *C. botulinum* spores or substances with high spore counts can be intentionally added to foodstuffs and feedingstuff and, if favorable conditions exist, BoNTs can be formed before consumption occurs. The *C. botulinum* spores in foodstuffs and feedingstuff can, under favorable conditions, trigger in vivo formation of BoNTs by toxicoinfection. Clinical reports have shown that botulism occurs in livestock and some outbreaks in ruminants have been associated with feedstuff containing carrion, chicken litter, bakery waste, ensiled chicken litter, and in horses and ruminants consuming alfalfa cubes and wet (high water activity, a_w) anaerobic feedingstuff packed in plastic, respectively (Kinde et al., 1991; Braun et al., 2005; Lindstrom et al., 2010). Spores of *C. botulinum* can be excreted in milk or milk may be contaminated during milking, and there is evidence that BoNTs are excreted in milk (Bohnel and Gessler, 2013).

61.3.1.2 Mechanism of action

BoNTs, after internalization by the cholinergic nerve terminals, cleave snare proteins and thereby block docking of the vesicles, thus preventing the release of acetylcholine from the presynaptic membrane (Pirazzini and Rossetto, 2017). Clinical signs of botulism are weakness, tremors, recumbency, laryngeal paresis, and other signs of nervous system dysfunction (Braun et al., 2005). For further details on toxicity of botulism, readers are referred to Gupta (2018).

61.3.1.3 Potential production and use

Spores of *C. botulinum* and BoNTs can be produced and/or incorporated into ingredients used in feedingstuff. Carrion, providing favorable incubation conditions exist, can serve as a substrate in feedingstuff for *C. botulinum* to grow and produce toxins. There is some evidence that insect larvae take-up and concentrate BoNTs and are unaffected by BoNTs (Hubalek and Halouzka, 1991). Insect larvae containing BoNTs can be used to contaminate animal feedingstuff and there is a trend to use insect larvae as a source of protein. The economics of a simulated terrorist attack by placing BoNTs in milk at a farm bulk tank or in a milk transport tank demonstrates in a "table top" model the "bow tie" far-reaching distribution effect with an estimate cost, including of human health care, of $8 billion (USD) (Wein and Liu, 2005).

61.4 Plant toxins

61.4.1 Toxins in seeds

Poisonous plants and their seeds could be used to adulterate feedingstuff (Keremidis et al., 2013). Seeds of poisonous plants have become incorporated into animal rations and have resulted in animal intoxication (Burrows and Tyrl, 2001). Grain screenings that contain high levels of toxic plant seeds and/or mycotoxins, could be distributed by a terrorist as a potential feed ingredient. Toxic seeds can be directly introduced into animal rations. The safety of screenings generally comes under government regulations. Screenings that do not meet regulations could be incorporated into animal feedingstuff as a terrorist tactic.

61.4.2 Castor beans (ricin)

61.4.2.1 Background

Castor beans (*Ricinus communis* L.) are grown commercially for castor oil, which is used in cosmetics, coatings, and also has industrial and automotive applications including biodiesel (Patel et al., 2016). There is approximately 1 tonne of residual cake containing ricin produced for every 0.5−0.6 ton of oil produced. Castor bean cake is sold as fertilizer, composted product (may be toxic if ingested), soil enhancers, and other uses, and in some countries it is used in limited quantities as a feed supplement. The castor bean and residual cake contain a cocktail of lectins that are not easily detoxified (Gomes et al., 2018). Ricin, a potent lectin in castor beans and residual cake, is soluble in water over a wide range of pH values. The ricin content of castor beans varies with the growing conditions and genetics of the plant. A general guide is a gram of castor beans contains 1−2 mg of ricin and ricin levels can be as high as 20 mg/g castor beans. Five

percent of the total protein in castor beans can be ricin, and biologically active ricin is present in the waste mash (castor cake) after the oil has been extracted. The ricin content of the extracted mash generally is 1%–10% (w/w). Ricin should be considered relatively heat-stable at temperatures used for food processing (Audi et al., 2005; Janik et al., 2019). The seed husks of *R. communis* are also poisonous and the recognized tolerable level is 10 mg of seed husks per kilogram of feed. A fatal dose in cattle is estimated to be 0.5 mg/kg body mass (Alexander et al., 2008). The ricinine piperidine alkaloid, a toxin of lesser potency, is found in all parts of the castor plant and is heat-stable. Ricinine stimulates the central nervous system (Ferraz et al., 1999).

61.4.2.2 Weaponization of ricin

Ricin has been used to commit murder and kill animals. It captures the interest of both terrorist organizations and agencies involved in protecting populations from terrorists. It is available in larger quantities using inexpensive technologies to extract it in its crude form from castor seed meal, a byproduct that has a low sale value. Ricin generally is not considered a weapon of mass destruction but is considered a weapon for locational use. Its distribution in a common feedingstuff ingredient could have substantial consequences. Some authors consider ricin a weapon more likely to be chosen by a "lone wolf" type of terrorist with an understanding of extraction and distribution technology.

61.4.2.3 Toxicity and mechanism of action

The seed coat of the castor bean needs to be fractured for the ricin to be released (Burrows and Tyrl, 2001). Ricin in extracted mash or cake is biologically available. Most animal species, including birds, are sensitive to castor bean poisoning. The severity of intoxication depends on the dose of ricin, and with ingestion of seeds, the number of seeds ingested and the liberation of ricin by degradation of the seed coat by some form of mastication. Ricin is absorbed from the gastrointestinal tract. The estimated lethal dose of castor bean seeds by species is given in Table 61.1. It is likely that the lethal dose for some species is lower than the doses found in the literature. Due to degradation in the rumen, mature ruminants are considered to be more resistant to ricin than are young ruminants and monogastrics.

Ricin is a lectin-type globular glycoprotein. It is a type-2 ribosome-inactivating protein (RIP) with A and B chains (Audi et al., 2005; Bolognesi et al., 2016; Janik et al., 2019). Ricin is absorbed from the gastrointestinal tract. Cells have a slow uptake of ricin and this can explain the lag time between exposure and onset of clinical signs. The ricin B chain attaches to the surface of the plasma membrane at the galactose-containing

TABLE 61.1 Sensitivity of different species to castor bean intoxication by oral exposure.

Species	Lethal[a] castor bean mass/ kg of body mass	Comment
Horse	7–240 mg	Considered the most sensitive species
Human	225 mg	Dose altered by the degree of mastication
Duck	0.7–1.2 g	1/8 duck died at 16 days after treatment. Grinding of the seeds by the proventriculus can be a variable in determining toxicity
Chicken	10–14 g	Grinding of the seeds by the proventriculus can be a variable in determining toxicity and values could be considerably lower for macerated seeds
Goose	400 mg	Grinding of the seeds by the proventriculus can be variable in determining toxicity
Rabbit	1.2 g	Mastication considered to be required
Pig	1.2 g	Mastication is required; 0.5 g husks/kg body mass is also fatal
Goat	5 g	Age of ruminant can be a factor as the rumen microbes inactivate ricin. Inactivation by the rumen and degree of mastication of seeds are factors in toxicity.
Sheep	1–2 g	Age of ruminant can be a factor as the rumen microbes inactivate ricin; 1.4–2.8 mg ricin-containing cake fed for 8 months and caused intestinal pathology
Cattle	1–2 g	Age of ruminant can be a factor as the rumen microbes likely inactivate ricin. Considered to be more resistant to intoxication than sheep

[a]*It is likely that the lethal dose for some species is lower than the doses found in the literature.*
Source: Adopted from Burrows, G., Tyrl, R., 2001. Toxic Plants of North America. Iowa State University Press: Ames, IA.

glycoproteins, pits are formed, and ricin is internalized by endocytosis and forms an intracellular vesicle. The vesicles containing ricin coalesce and form endosomes. The internalized ricin can dissociate from the endosomes and be transported to lysosomes for degradation or the vesicles can be removed from the cell by exocytosis. Ricin can bleb off the endosome and return to the cell surface by vesicular and tubular structures, or it may be transported to the Golgi apparatus. In the Golgi apparatus, the A chain undergoes retrograde transport to the endoplasmic reticulum. Cleavage of the A and B chains occurs here. From the endoplasmic reticulum, the ricin A chain is translocated to the cytosol. Here, the ricin A chain catalytically inactivates ribosomes. The A chain is an enzymic polypeptide that catalyzes the N-glycosidic cleavage of adenine from 28S rRNA loupe in the 60S ribosomal subunit. The removal of adenine from 28S rRNA in the ribosome destroys its functionality and thereby blocks protein synthesis. One ricin A chain can enzymatically inactivate 1500 ribosomes/min. Using other mechanisms, ricin causes the release of inflammatory mediators. Ricinine is a central nervous system stimulant and is theorized to, in the nervous system, inhibit the postsynaptic γ-aminobutyric acid receptor subtype A and to alter the release of glutamate. It is excreted in the urine and if identified in serum and urine is a biomarker of castor bean intoxication. The only treatment for ricin intoxication is supportive care.

61.4.2.4 Analytical methods

Analytical methods used to identify ricin include immune methods (ELISA) and liquid chromatography/mass spectrometry (LC/MS) (Duracova et al., 2018). Gastric contents can be assayed, and ricin can be detected in blood and body fluids by radioimmunoassay and LC/MS (Mouser et al., 2007). Ricinine levels can be assayed in serum and urine using LC/MS (Roen et al., 2013). Polymerase chain reaction methods are available to detect the DNA in castor beans.

61.4.2.5 Clinical and pathological findings

Clinical signs of ricin intoxication from ingestion are high morbidity, abdominal pain, emesis, diarrhea, and lethargy leading to death. Hematemesis and melena may be observed. Clinicopathology includes elevated hepatic enzymes in serum, hyperphosphatemia, and sometimes hypoglycemia. Electrolyte imbalance can occur because of diarrhea and emesis. Pathological findings in a dog were hepatocellular necrosis with fibrosis of central veins and sinusoids, and lymphocytic infiltrate (Mouser et al., 2007). Jejunal mucosa can be eroded and infiltrated with leukocytes, and the tunica muscularis can also be affected. Hemorrhage can be observed in the mucosal and muscular layers. Necrosis can be seen in the spleen. Renal pathology has also been reported.

61.4.3 Other plant source type 2 RIPs

Abrin is a plant source type 2 RIP and is a candidate for weaponization (Chaturvedi et al., 2015). It is found in *Abrus precatorius* (also known as rosary pea, Indian licorice, jequirity bean). The toxicology of abrin is considered to be very similar to ricin. The seeds of *A. precatorius* are not used as oilseed but are used in ethnic medicine. A similar *Abrus* toxin is pulchellin, produced by *Abrus pulchellus*. The rosary pea has been reported to be more toxic than castor beans. Like castor beans, the rosary pea must be masticated to release the toxins (Burrows and Tyrl, 2001). Species sensitivity is variable, and horses are considered to be the most sensitive. The mature goat is considered to be a more resistant species, and seed in the form of 2 g/kg body weight is reported as a lethal dose. The reported lethal dose of seeds for the horse is approximately 100 mg/kg body mass and for cattle it is reported to be 600 mg/kg body weight. This observation provides some evidence that abrin is denatured in the rumen.

61.5 Rapidly acting and easily available substances

61.5.1 Cyanide

Cyanide (CN^-) is considered a terrorist weapon by the oral and inhalation routes of exposure and can be added to feedingstuff and water. It has a history of use as a fumigant and in controlling problem wildlife, and unwanted feral "domesticated" animals. It also has alleged use as an agricultural terror agent in livestock drinking water. A common feed ingredient laced with cyanide could have wide distribution from a single source. Cyanide is also considered a low-technology terrorist weapon for a "lone wolf" or a terrorist group.

61.5.1.1 Mechanism of action

The toxicology of CN^- is complex. The lethal effect of cyanide is blockage of cytochrome C oxidase and loss of electron transfer, and the end result is that molecular oxygen is not utilized. In addition to cytochrome C, other cytochromes are adversely affected by forming complexes with cytochrome iron. The oxidized cytochrome−CN complexes are stable. However, the complex is relatively unstable in a chemically reductive environment and creating an endogenous reductive environment is a mechanism of action for antidotal agents.

61.5.2 Insecticides and drugs

To adulterate animal feedingstuff and poison animals, terrorists could use licensed insecticides and drugs. Large numbers of cattle and other domestic livestock, including poultry, have been contaminated and poisoned with insecticides that were inadvertently and forensically incorporated into feedingstuff (Neher, 1999; Guitart et al., 2010). Coordinated efforts are required to diagnose, contain, and eliminate insecticide- poisoned and contaminated animals from the food web, including feedingstuff obtained from rendered animals. There have been instances in which transport devices have been used to transport and temporally store insecticides, and treated seeds, and then subsequently used for silage and other feedingstuff. These activities resulted in livestock poisonings and contamination. The use of insecticides that are persistent organic compounds (POCs) for terror actions may also present a residue problem in the surviving animals and may require a huge input of governmental and other resources to prevent and remove contaminated edible products from the foodstuff and feedingstuff pipelines. The toxicology and treatment of insecticide poisoning have been discussed in detail in Gupta (2018). Drugs licensed for specific indications and species, can be used to adulterate feedingstuff and cleaning-sanitizing agents. Stafford et al. (2018) reported the addition of fipronil, a broad-spectrum insecticide used as a treatment for ectoparasites in companion animals, which was intentionally added to the cleaning product DEGA-16. This incident was not linked to terrorism. The altered DEGA-16 containing fipronil, used to control red mites (*Dermanyssus gallinae*) that affect chickens, was sold to chicken producers and egg producers in 16 countries including Switzerland and China (EU, 2017). The EU Rapid Alert System was activated because of illegal residues in poultry-sourced foodstuffs (EU, 2018). The damage was estimated at over $39 M with 258 companies unable to sell products during the foodstuff recall period. The estimated loss did not include the impact on markets and reputation. Also, because of this incident, foreign egg producers penetrated markets located in the European Union. This incident shows that residues of illegal substances in edible animal products can have large consequences and trigger regulatory action to protect public health. This incident shows that terrorists can easily adulterate a product resulting in huge financial consequences.

61.6 Persistent organic compounds

61.6.1 Background

POCs, also known as POPs (persistent organic pollutants), could be candidates for a terrorist attack on livestock. Most POCs are biomagnified in the food web. The health, economic, and political impact from their use in a terrorist attack would likely be huge financial loss, including health care, based on the impacts from reported inadvertent contaminations of feedingstuff (Carter, 1976; Kay, 1977; Reich, 1983; Terrell et al., 2015; Malisch, 2017; Walker et al., 2019). The majority of POCs are bioconcentrated in body lipids. Most of these compounds require high doses to cause acute illness in humans, livestock, poultry, and fish. At low doses the resultant illness in food-producing animals is generally less evident and these animals and their products are used as foodstuffs. The human health issues from long-term exposure to POPs has increasing recognition. There are residues in edible animal products and rendered animal products used in animal feedingstuff. The occurrences of POCs contaminating feedingstuff and feed ingredients are underappreciated (Kim et al., 2007). The lack of specific clinical signs and lesions increases the risk of nonrecognition and contaminated animals passing slaughter inspections, and subsequently their products being consumed by humans and their byproducts being used in feedingstuff. For the POCs, the safety of foodstuffs and feedingstuff are reliant on chemical analyses (Kim et al., 2007). Occurrences of POCs being relayed from contaminated animal-source foods to humans and their byproducts to feedingstuff are known (Kay, 1977; Reich, 1983; Fries, 1985; Huwe and Smith, 2005; Kim et al., 2007; Covaci et al., 2008).

Historical incidents are discussed to show the economic and health impacts that can be caused by feedingstuff becoming contaminated with POPs. Reich (1983) reported that from 1969 to 1974, excluding the Michigan polybrominated biphenyls (PBBs) incident, the dollar loss to livestock and poultry producers by identified chemical contamination was $21.5 million. The PBB incident in Michigan, likely the largest in US history, is given as an example. The cause of this incident was a PBB flame-retardant (approximately 200−400 kg), intended for use in plastic, which was shipped to a feed mill instead of magnesium oxide. The PBBs were subsequently mixed into livestock feedingstuff (Carter, 1976; Reich, 1983). More than 40 years later, the health effects of this incident are being elucidated, demonstrating the long-term multigenerational impact on human health, and the long-term healthcare-linked costs which have never been estimated (Small et al., 2011; Terrell et al., 2015; Jacobson et al., 2017; Walker et al., 2019). Environmental sources of POCs can be introduced into feedingstuff and require regulatory responses. Clay mineral, contaminated with polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs), was introduced into mineral mixes. Other incidents of contaminated clay clearly show that feedingstuff contamination can occur over a substantial period of time before the contamination is recognized and

foodstuffs and feedingstuff are protected by regulatory action (Hayward et al., 1999; Hoogenboom et al., 2010). Orange peel, bakery waste, recycled fat, choline chloride, and zinc oxide can all be used as feed ingredients and these products have been contaminated with POPs, resulting in contamination of animal-source foodstuffs (Malisch and Kotz, 2014). Proactive analytical surveillance for POPs requires substantial resources consisting of highly trained personnel, sophisticated laboratory procedures, and infrastructure to meet laboratory safety requirements. The analytical and personnel support for investigations into POCs contaminating livestock feedingstuff can challenge budgets, quarantines can impose harsh economic consequences on livestock and poultry producers, and there can be political pressure on regulatory agencies to restrict or delay investigations (Reich, 1983; Deshingkar, 2002). Some of the highest levels of POPs in consumed foodstuffs have occurred because of feedingstuff being contaminated. The health impacts of POPs in the human diet are likely underappreciated and there is evidence to show the effects can be multigenerational (Blanck et al., 2000; Kim et al., 2007; Walker et al., 2019).

61.6.2 Potential economics of terror attacks using persistent organic pollutants

The history of incidents clearly shows that feedingstuff contaminated with POCs has caused large economic losses and impacts on animal and human health. Most POCs are biomagnified in the food web. If feedingstuffs have a wide distribution, then the measurable economic impacts and regulatory costs can be immense. In the Michigan (US) PBB disaster, 500 farms were under quarantine (Carter, 1976). More than 865 tons of feedingstuff, 29,800 head of cattle, 5920 hogs, 1470 sheep, 1.5 million chickens, 34,500 kg of dairy products, and 5 million eggs were destroyed (Carter, 1976). The total cost was estimated at more than $215 million at 1983 currency value and this loss did not include human health (Reich, 1983). In the ball clay incident, it was estimated that 1.7 million eggs per day (at that time approximately 1% of the US egg production) and 35% of the farm-reared catfish were contaminated with PCDDs/DFs (Hayward et al., 1999). These animal-source foodstuffs, contaminated with PCDDs/DFs, were consumed by humans (Fiedler et al., 1997; Rappe et al., 1998). In the United States, more than 400 upright silos were coated with a PCB-containing resin that migrated into silage. The livestock and their edible products were contaminated with PCBs (Sweeney et al., 2001). In Belgium, a tank of animal fat (animal feed ingredient) feed was contaminated in January 1999 with a PCB (congener closely matched Aroclor 1260/1254) containing PCDDs/DFs and caused an unprecedented feedingstuff and foodstuff crisis (Lok and Powell, 2000). It is estimated that 150 kg of PCBs with PCDDS/DFs contaminated the recycled animal fat. Nine feed manufacturers used the contaminated oil to formulate poultry and other livestock feedingstuff. There was a considerable lag time for the government to engage regulator action. Several countries banned imports of animal source foodstuffs and products containing animal-sourced ingredients. Estimates losses from this disaster are US $1.5 billion (Lok and Powell, 2000). No estimates have been made regarding the human healthcare cost of these incidents.

61.7 Heavy metals and metalloids

Heavy metals and metalloids have been used forensically to maliciously poison livestock and nonforensic incidents of lead poisoning occur. Arsenic has a history of weaponization (Li et al., 2016). Lead is readily available to terrorists, for example, from discarded lead batteries. Humans can be exposed to heavy metals in meat-stuffs and milk from domestic animals.

61.7.1 Lead

Lead is readily available in the form of spent lead acid batteries and lead gunshot. Pulverized lead is not rapidly identified in animal feedingstuff, allowing a successful terrorist attack. Lead poisoning incidents with lead-acid batteries, lead gunshot, and environmental sources account for the majority of cattle poisonings in North America. Lead is excreted in milk, deposited in bones, liver, and kidney, and is considered a food safety issue (Coppock et al., 1991). Cattle that are asymptomatic for plumbism can excrete lead in milk (Bischoff et al., 2014). Lead poisoning is likely underdiagnosed and can be confused with thiamin-responsive polioencephalomalacia in cattle (Coppock et al., 1991). The favorable clinical response to thiamine therapy could result in failure to diagnose plumbism. Lead is solubilized by silage fluids and migrates in the liquid phase of silage (Coppock et al., 1988; Bischoff et al., 2014). A small amount of lead can poison a large number of livestock. Lead isotope profiling can be used in forensic investigations to identify the source of lead (Buchweitz et al., 2015).

61.7.2 Arsenic

Arsenic is used to maliciously poison livestock. Arsenic can be mixed with feedingstuff and water, or just made available to cattle. Cattle voluntarily consume arsenic (Schild et al., 2019). Arsenic is used in agrocrime and has been used for crimes of revenge. In acute arsenic poisoning, survival is generally low. Arsenic could have been used in acts

of terrorism targeting cattle (Kosal and Anderson, 2004). Arsenic could be used by a "lone-wolf" terrorist.

61.8 Concluding remarks and future directions

Feedingstuff is essentially unprotected from chemical adulteration by agrocrime and terrorist attack. The psychological and sociological profiling of terrorist and terrorism-orientated groups involved suggest that feedingstuff is more likely to be attacked by small groups and "lone-wolf"-type terrorists. Historical review of feedingstuff being contaminated with chemicals, especially persistent organic pollutants, has shown that the costs can be billions of dollars, exclusive of human health impacts, and these incidents have not been linked to terrorism. A simulation using a terrorist attack on milk using BoNTs showed that a primary estimated cost of $8 billion would primarily be in human health care (Wein and Liu, 2005). Forty years after a PPB incident in Michigan (United States), multigenerational human health impacts are being identified. Recognition of chemical contamination of feedingstuff and rapid responses are required to protect foodstuffs. The European Union has the RASFF (Food and Feedingstuff Safety Alerts) for consumer protection. Feedingstuff could be contaminated as collateral damage from other acts of terrorism, for example wildfires, building fires and fire runoff water, and dirty bombs. More emphasis and study need to be placed on feedingstuff as a target for terrorists. Feedingstuff can be targeted without the affected animals showing overt signs of illness and with chemical resides in edible animal products and rendered animal products. Identification of chemical adulterants in feedingstuff requires substantial infrastructure in trained personnel, equipment, and laboratory facilities. The future direction is to fully recognize the one-health−one-medicine concept that has to be used in the management of chemical and radioisotope contamination of feedingstuff. Laboratory capacity to analyze feedingstuff and foodstuff must be developed and maintained. Terrorist attacks outside the agri-food sector can result in collateral chemical and radioisotope contamination of animal feedingstuff and this must be included in the emergency plan to prevent consumption of chemically contaminated foodstuffs.

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Chapter 62

Chemical warfare agents and risks to animal health

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62.1 Introduction

Animals are susceptible to all four basic types of military agents: choking (such as chlorine gas and phosgene), blister (such as mustard, lewisite, and phosgene oxime), blood [such as cyanide and hydrogen cyanide (HCN)], and nerve agents [such as tabun, sarin, soman, and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX)]. They can also be affected by incapacitating agents such as 3-quinuclidinyl benzylate (BZ), riot control agents (RCAs), ricin, and abrin. Since chemical warfare agents (CWAs) can be deployed by a variety of inexact methods (including bombs, spray tanks, rockets, missiles, land mines, and artillery projectiles), domestic and wild animals living in proximity to human populations can be affected (USACHPPM, 2001a). Americans are estimated to have 89.7 million dogs, 94.2 million cats, and 7.6 million horses (Bedford, 2019). In addition to pets, livestock are very important potential targets of attack as they have secondary ramifications for human health and disruption of the food chain.

The Centers for Disease Control and Prevention (CDC) Strategic Planning Working Group, as part of their preparedness plan for possible terrorist attacks using biological or chemical weapons, has called for "prompt diagnosis of unusual or suspicious health problems in animals" (Anonymous, 2000a). The CDC recommended establishing "criteria for investigation and evaluation of suspicious clusters of human and animal disease or injury and triggers for notifying law enforcement of suspected acts of chemical terrorism." With many of the military agents, there are few initial indicators of a chemical attack. It has been proposed that animals could serve as sentinels for chemical terrorism. These animals would be similar to the canaries used by coal miners in the United Kingdom and the United States to provide early warning of deadly mine gases.

Throughout history, it has been noted that during chemical warfare attacks, animals may also be affected. A newspaper article from World War I gives details of the effects on animals during an unspecified type of gas attack (Anonymous, 1918):

> *horses suffer much from the noxious fumes, and are subsequently thrown into a state of nervous terror on again scenting them. Mules are more inclined to stand their ground, and appear as if trying not to breathe . . . Cats quickly scent the gas, and run about howling. Guinea pigs are first to succumb . . . Rats and mice emerge from their holds, and are found dead in quantities, which as the soldiers say, is the only advantage of a gas attack by the enemy.*

The soldiers believed that different species of animals may be more sensitive to certain types of gas attacks than humans:

> *Poultry of all kinds are useful for giving warning, ducks and fowl becoming agitated 10 min or so before the oncoming gas clouds. . . . Only the sparrow seems to disregard the poisonous vapor, and sparrows chirp on where horses are asphyxiated, and bees, butterflies, caterpillars, ants and beetles die off in great numbers. The gas at once kills snakes, and earthworms are found dead in their holes many inches below the ground.*

Not much has changed in using animals as sentinels over the years. Crates of rabbits were placed on the cargo deck of ships transporting nerve gases during World War II, and the crew were instructed to watch for sudden animal deaths that could signal a gas release (Brankowitz, 1987). Even today, with the development of sophisticated biosensor technology, the use of animals as sentinels continues to

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be explored (Paddle, 1996). The US Environmental Protection Agency (EPA) is now considering evidence regarding the use of animals as sentinels for chemical threats (EPA, 2006).

Some of the CWAs that may pose the greatest risks to animal health are described next. For information on their mechanism of action, readers are referred to Section II of this book.

62.2 Chemical warfare agents

62.2.1 Chlorine gas

62.2.1.1 Clinical signs

Chlorine gas is very irritating, and in concentrated amounts, it can even be corrosive. The eyes, skin, nose, throat, and mucous membranes can all be affected. Inhalation can lead to rhinorrhea, dyspnea, hypoxemia, ataxia, syncope, muscle weakness, tachypnea, laryngospasm, bronchospasm, pneumonitis, pulmonary edema, and death (Noe, 1963; Evans, 2005).

Ocular exposure can result in severe pain and blindness. Dermal exposures cause erythema and pain, and high concentrations of chlorine gas can cause dermal burns (Raffle et al., 1994).

62.2.1.2 Kinetics

Respiratory, dermal, and ocular irritation starts immediately. The speed of onset and severity of signs is directly related to the concentration (Bingham et al., 2001). Acute lung injury peaks in 12−24 h. With mild exposure, signs disappear within 6 h, but can continue for more than 24 h with severe exposures. Death usually occurs within 48 h (Decker, 1988). Exposure to moderate or severe concentrations can result in chronic respiratory dysfunction (Decker, 1988). This can be career- or life-ending if the affected animal is an equine athlete or a working dog.

62.2.1.3 Decontamination and treatment

Move animals into fresh air and onto higher ground. Monitor respiratory rates and oxygenation status ($Sp\mathrm{O}_2$). If coughing or dyspnea develops, provide supplemental oxygen and ventilation. Bronchodilators should be used to counteract bronchospasm (Wang et al., 2004). Sedation and pain control may be needed so that the animal can be handled safely.

Corticosteroid use is controversial. Animal models have shown positive results; however, administration to humans has not been shown to provide any significant change (Traub et al., 2002; Chen et al., 2013). Multiple other therapies have been used in laboratory situations to decrease lung injury: antioxidants (McGovern et al., 2010; Zarogiannis et al., 2011; Wigenstam et al., 2015), cAMP-elevating agents (Hoyle et al., 2016), heparin (Zarogiannis et al., 2014), and nitric oxide modulating agents (Honavar et al., 2017).

For ocular exposure, the eyes should be flushed with generous amounts of tepid 0.9% saline or tap water for at least 15 min. After flushing, fluorescein should be used to stain the eyes and check for corneal ulcers (Grant and Schuman, 1993). Bathing with dish soap and water will remove chlorine from the skin and fur or feathers.

Chlorine does not leave an environmental residue, so animals may be returned to affected pastures within hours to days, and more quickly in warm environments (Munro et al., 1999). When entering the area contaminated with chlorine gas, rescuers should wear self-contained breathing apparatus (SCBA) and protective clothing (i.e., gloves, gowns, and masks) until the gas dissipates. The risk for secondary contamination of rescuers is low. Chlorine gas does not bind to leather or fabrics.

62.2.1.4 Species susceptibility

Chlorine gas is heavier than air and will settle in low areas. Pets and smaller livestock may be more at risk than humans due to their proximity to the ground. There are no controlled studies showing that animals are more sensitive to chlorine gas or that they will develop signs sooner than humans.

62.2.2 Phosgene

62.2.2.1 Clinical signs

Most animal exposures to phosgene are from inhalation, but there can also be dermal exposures to the liquefied material. The severity of pulmonary injury correlates with the concentration and length of exposure, and initial symptoms are not always a good indicator of prognosis (Diller, 1985; Bingham et al., 2001). Dyspnea, cough, cyanosis, and hemoptysis can progress to hypoxemia and hypoventilation (Borak and Diller, 2001). Death is from anoxia secondary to pulmonary edema (Borak and Diller, 2001; Proctor and Hughes, 2004).

Animals may develop secondary organ damage from anoxia. Dogs experience bradycardia, followed by tachycardia and progressive hypotension with severe phosgene poisoning (Patt et al., 1946). With concentrations greater than 200 ppm, phosgene can enter the blood and cause hemolysis and coagulopathies (Sciuto et al., 2001). Direct contact with liquid phosgene can cause dermal burns (Proctor and Hughes, 2004). Ocular exposure to both liquid phosgene and highly concentrated phosgene gas can cause severe eye irritation and corneal opacification (Grant and Schuman, 1993; Proctor and Hughes, 2004).

Prognosis is directly related to the severity of pulmonary injury. Animals that survive the first 24–48 h still have a guarded prognosis. These animals are more susceptible to infectious agents, as they have suppressed natural killer cell activity. Infections may become evident 3–5 days after exposure. Animals may develop chronic exercise intolerance and abnormal pulmonary function (Borak and Diller, 2001). Working dogs and horses may no longer be able to fulfill their functions.

62.2.2.2 Kinetics

Respiratory signs develop 2–6 h postexposure in most patients, but can be delayed up to 15 h with exposures to lower concentrations (<3 ppm) (Borak and Diller, 2001). Concentrations of 3–5 ppm cause immediate conjunctivitis, rhinitis, pharyngitis, bronchitis, lacrimation, blepharospasm, and upper respiratory tract irritation. Extended (170-min) exposure was fatal (Diller 1985; Proctor and Hughes, 2004). Exposure to 50 ppm for 5 min or longer will cause pulmonary edema and rapid death (Chemstar, 1996a,b; Borak and Diller, 2001; RTECS, 2008). If the animal survives, pulmonary edema begins to resolve in 2–3 days.

62.2.2.3 Decontamination and treatment

Move animals to fresh air and higher ground. Bathe animals with soap and water and flush eyes for 15 min with tepid water or 0.9% saline. Animals should be monitored for 24 h for the development of pulmonary edema (Borak and Diller, 2001). If animals are coughing or dyspneic, administer 100% oxygen. Intubate and ventilate as needed. Patients with pulmonary edema should be managed the same as an acute respiratory distress syndrome (ARDS) patient (i.e., mechanical ventilation with oxygen and positive end-expiratory pressure). The mechanism of phosgene-induced lung injury is complex and not fully understood. Many treatments have been tried including bronchodilators (Kennedy et al., 1989), antiinflammatories (Guo et al., 1990; Chemstar, 1996a,b), xanthine derivatives (Zhang et al., 2010), antioxidants, ACE inhibitors, and TRP inhibitors (Sciuto et al., 1995; Holmes et al., 2016) to reduce injury. Intravenous fluids should be used for cardiovascular support, but monitored for overhydration. Colloids are preferred over crystalloids, as they will remain in the vascular space for a longer period of time. Oxygen supplementation will resolve most of the arrhythmia.

Phosgene is nonpersistent in the environment. Moisture reduces air concentrations (Borak and Diller, 2001). The potential for secondary contamination of rescue personnel is low, but rescuers should wear proper protective clothing. Phosgene gas does not persist in fabric or leather.

62.2.2.4 Species susceptibility

Phosgene is heavier than air and will settle close to the ground. This can affect species that are low to the ground or that are pastured in low-lying areas. There is no indication that animals are affected before or at lower levels than their human counterparts.

62.2.3 Mustard gas

62.2.3.1 Clinical signs

Mustard gas is a vesicant that is toxic by all routes of exposure (oral, inhalation, dermal, and ocular) (Sidell et al., 1997; Pohanish, 2002). Mustard causes both localized and systemic cellular damage, and tissues with high cell turnover are the most affected (NATO, 1973). Mustard gas can produce erythema, severe pruritus, blistering, ulceration, and necrosis of exposed skin (Borak and Sidell, 1992; Dacre and Goldman, 1996; Budavari, 2000; Pohanish, 2002).

Ocular exposure can cause pain, lacrimation, corneal ulceration, swelling, blepharospasm, and blindness (NATO, 1973; Borak and Sidell, 1992; Dacre and Goldman, 1996; Garigan, 1996). Pathognomonic signs of mustard gas poisoning include porcelain-white areas in the episcleral tissues and the formation of large, varicose veins (Grant and Schuman, 1993). Conjunctivitis and keratopathy can be seen chronically after exposure (Grant and Schuman, 1993).

Inhalation of small amounts of mustard gas produces nasal discharge, sneezing, epistaxis, and coughing within 12–24 h of exposure. Higher concentrations or longer exposures can cause pulmonary damage, hypoxia, and respiratory acidosis. Seizures may be seen with acute high doses (Sidell et al., 1997).

Mustard gas is also a radiomimetic (Sidell et al., 1997). It destroys precursor cells in the bone marrow, leading to leukopenia, thrombocytopenia, pancytopenia, and anemia (Borak and Sidell, 1992; Dacre and Goldman, 1996). Infection can be seen secondary to bone marrow damage (Sidell et al., 1997). Bone marrow aplasia and death can be seen in severe cases.

62.2.3.2 Kinetics

Both liquid and vaporized mustard have rapid skin penetration. The higher the dose, temperature, and humidity, the quicker the absorption (NATO, 1973). Mustard is dermally absorbed through hair follicles and sweat glands within minutes. Cellular reactions begin within 1–2 min of mustard making contact with skin or mucous membranes, but clinical effects are delayed between 2 and 24 h (Grant and Schuman, 1993; Sidell et al., 1997).

The skin initially pales and then becomes erythematous within a few hours of exposure (Requena et al., 1988).

Erythema, blisters, bulla, and small vesicles form over 2−24 h. The blisters can progress for several more days. Erythema resolves over 3−7 days, while ulcers take 6−8 weeks to heal (Garigan, 1996; Sidell et al., 1997). Discoloration (brown or black hyperpigmentation) commonly occurs after resolution of the burns, especially in areas with thinner skin (Requena et al., 1988).

Ocular absorption happens within minutes. Clinical signs begin within 1−12 h depending on the concentration (Requena et al., 1988). Inhalation produces respiratory signs (rhinorrhea, sneezing, epistaxis, and coughing) within 12−24 h of exposure. A severe exposure produces a productive cough, tachypnea, pulmonary edema (rare), and pulmonary hemorrhage within 2−4 h. Ingestion of small amounts can cause hypersalivation and vomiting within 24 h, while larger amounts can cause gastrointestinal bleeding (rare) and bloody diarrhea within 3−5 days.

Mustard preferentially accumulates in fatty tissue (Somani and Babu, 1989). Mustard is excreted in the urine over 72−96 h after i.v. administration in rats and mice (Dacre and Goldman, 1996). Complete blood count (CBC) changes are not evident for 3−5 days postexposure. Leukopenia usually occurs between days 7 and 10 (Garigan, 1996).

62.2.3.3 Decontamination and treatment

Animals should be moved into fresh air. Emesis is not recommended, and activated charcoal administration after oral ingestion is controversial. Sodium thiosulfate (2% solution) given orally may help in cases of oral exposure (Borak and Sidell, 1992; Dacre and Goldman, 1996). Perforation and stricture formation can follow esophageal burns.

For ocular exposures, flush eyes with tepid water for at least 15 min. Follow with 2.5% sodium thiosulfate ophthalmic to help neutralize the mustard. The eyes need to be decontaminated quickly, as late flushing of the eye provides no benefit (Sidell et al., 1997). Topical ophthalmic antibiotics and pain control should be used if corneal lesions are present (Sidell et al., 1997).

Animals need to be bathed with copious amounts of soap and water. If dermal decontamination is not implemented quickly, mustard will react with the skin and cannot be easily removed (Sidell et al., 1997). Sodium thiosulfate (2.5% solution) can be used dermally to neutralize mustard exposures (Garigan, 1996). Animals may also be bathed with dilute (0.5%) hypochlorite solutions (Borak and Sidell, 1992). Monitor for dermal burns. Secondary infection is common. Topical silver sulfadiazine can be applied to all burns, and an Elizabethan collar used to decrease ingestion of the ointment and self-trauma. All equine and ovine patients should be inoculated with tetanus toxoid. Vaccination of other species should be determined on a case-by-case basis.

Monitor for respiratory irritation. If seen, monitor arterial blood gases and pulse oximetry. Thoracic radiographs may be taken, but there can be a lag time of up to 2 days before infiltrates are seen (Smith, 1999). Nebulization with 2.5% sodium thiosulfate or *N*-acetylcysteine may help neutralize the mustard gas (Garigan, 1996). Provide oxygen, ventilation, and inhaled beta agonists if needed. Dexamethasone, promethazine, vitamin E, melatonin, nitric oxide synthase inhibitors, protease inhibitors (such as doxycycline), and surfactant replacement have all shown beneficial effects against mustard gas poisoning in laboratory animals (Requena et al., 1988; Weinberger et al., 2011). Anticoagulants and fibrinolytics may help reduce bronchial exudates and fibrin casts (White et al., 2016).

Serial CBCs with platelets should be monitored for 2 weeks after exposure. Antibiotics should be given if leukopenia develops (Sidell et al., 1997). Mustard can be detected in urine and body tissues for up to 1 week post-exposure using gas chromatography-mass spectrometry (GC-MS; Vycudilik, 1985). This can confirm the diagnosis but is not likely to be of value in the management of the patient.

Mustard is persistent in the environment; it may remain for up to 1 week in temperate areas. It disappears in about 1 day in hot climates and in desert conditions. Since mustard binds to vegetation for days to weeks, grazing animals need to be kept away from these areas (USACHPPM, 2001b).

Rescue personnel must wear protective clothing, eye protection, and a respirator, as the potential for secondary contamination is high (HSDB, 2008). Mustard gas will penetrate wood, leather, rubber, and paints.

62.2.3.4 Species susceptibility

Dermal absorption of mustard varies greatly by species, and fur can be protective (Smith et al., 1997). However, rats absorb about 75% of a dermal dose through their skin, while only 20% is absorbed through human skin (Smith, 1999). The rat dermal LD_{50} is only 5 mg/kg, while the mouse and human dermal LD_{50}s are 92 and 100 mg/kg, respectively (RTECS, 2008). With oral dosing, humans appear to be much more susceptible (oral LD_{50} 0.7 mg/kg for humans, and 17 mg/kg for rats). Due to these variable results, more studies are needed to determine if animals would make good sentinel animals.

62.2.4 Lewisite

62.2.4.1 Clinical signs

Lewisite is an arsenical compound that acts locally as a vesicant but also causes systemic effects including decreased adenosine triphosphate (ATP) production and

increased capillary permeability (Sidell et al., 1997; HSDB, 2008). Pulmonary edema or ARDS can develop (Sidell et al., 1997).

Signs can be seen when lewisite is contacted dermally, orally, ocularly, inhaled, or ingested. Dermal and respiratory exposures are seen most frequently. Lewisite causes dermal, ocular, and respiratory lesions similar to those caused by mustard gas. Exposure to lewisite is very painful. Both vapor and liquid lewisite can penetrate skin. Reddening of the skin is followed by tissue destruction (EPA, 1985a; Goldman and Dacre, 1989; Sidell et al., 1997; Pohanish, 2002). Severe edema develops secondary to increased capillary permeability. Dermal burns are deeper than those seen with mustard gas and are quicker to appear (Goldman and Dacre, 1989; Sidell et al., 1997).

Ocular exposure causes immediate pain, lacrimation, and blepharospasm. Without rapid decontamination, within 1 min, permanent blindness may occur (EPA, 1985a; Pohanish, 2002). In livestock, this is a death sentence, as they cannot survive on the range when blind.

Inhalation of vapor causes irritation to the nasal passages, rhinorrhea, and violent sneezing (HSDB, 2008). Coughing and hemoptysis commonly occur (Sidell et al., 1997; HSDB, 2008). Arrhythmias and renal dysfunction are due to hypovolemia from fluid loss. After inhalation, dogs developed necrotizing pseudomembranous laryngotracheobronchitis (Goldman and Dacre, 1989). Death can occur within 10 min after inhalation of high concentrations (EPA, 1985a).

62.2.4.2 Kinetics

Immediate pain occurs upon inhalation, dermal, or ocular contact with lewisite. Skin penetration occurs within 3−5 min, especially following liquid exposures (Sidell et al., 1997). The skin becomes red, then gray, within 15−30 min after exposure (EPA, 1985a; Goldman and Dacre, 1989; Sidell et al., 1997; Pohanish, 2002). Severe blisters develop within 12 h. The blisters rupture about 48 h after occurrence, with large amounts of fluid seeping from the site. Healing is generally complete within 4 weeks.

Lewisite has extensive tissue distribution (HSDB, 2008). The highest concentrations were found in the liver, lungs, and kidneys in rabbits (> seven times blood concentration). The elimination half-life of arsenic in rabbits is 55−75 h (HSDB, 2008). For further details on toxicity of arsenic and lewisite, see Chapter 21.

62.2.4.3 Decontamination and treatment

Remove animals from affected areas. If any coughing or respiratory distress, monitor blood gases and SpO_2. Provide oxygen and assisted ventilation as needed. Nebulized beta agonists (and possibly corticosteroids) can be used to treat bronchospasm. Monitor electrolytes and packed cell volume (PCV), as there can be fluid shifts out of the vasculature (Goldfrank et al., 2002). Watch for liver and kidney failure.

As lewisite is a vesicant, emesis is not recommended in those species that can vomit (such as dogs, cats, swine, and ferrets). Dilution with milk or water is recommended. Activated charcoal is not helpful. Endoscopy can be performed very carefully to determine the extent of injury. Esophageal perforation, stricture formation, or both may occur.

The eyes should be flushed with copious amounts of tepid water for at least 15 min. If 5% dimercaprol [also known as *British antilewisite (BAL)*] ophthalmic ointment can be applied within 2 min, this may prevent a significant reaction. Application of up to 30 min after exposure will lessen the ocular reaction but will not prevent all damage (Goldfrank et al., 2002).

Animals should be washed with water and dilute household bleach (5% sodium hypochlorite) as soon as possible. Application of a 5% BAL ointment within 15 min can be effective in diminishing the blistering effects of lewisite (Smith, 1999). Remove BAL ointment with soap and water after 5 min. Leaving the ointment on too long can cause stinging, itching, or urticaria. Burns should be managed with pain control, antibiotics, and debriding as needed.

A chelator should be given if there is dyspnea, pulmonary edema, or skin burns larger than palm size (Goldfrank et al., 2002). BAL is the traditional arsenic chelator, but it has numerous side effects. The deep intramuscular injections are very painful and BAL can cause hypertension, tachycardia, and vomiting. 2,3-Dimercaptosuccinic acid (DMSA, Succimer) can also be used to chelate arsenic (Graziano et al., 1978). 2,3-Dimercapto-1-propanesulfonic acid (DMPS) is used in Europe and has been effective in protecting rabbits from the lethal effects of lewisite (Aposhian et al., 1982).

Lewisite remains in the environment for about 24 h, and it can react with water to form a solid arsenoxide that also has vesicant properties. Affected areas can be treated with strong alkalis to form less harmful substances. Rescue personnel need to wear protective clothing and masks, as the risk for secondary contamination is high. Carcasses should be disposed of properly, either buried deeply (away from water supplies), rendered, or incinerated to ensure safety of the food supply.

62.2.4.4 Species susceptibility

Lewisite has the potential to cause skin lesions in any species, but the risk is greatest in hairless animals, such as pigs, and decreases in fur-covered species (Smith et al., 1997). Mice and rats appear to be almost twice as

susceptible to dermal lewisite exposures as humans (LD_{50} equals 12, 15, and 30 mg/kg, respectively) (Sidell et al., 1997; DeRosa et al., 2002; RTECS, 2008). More studies are needed to determine if rodents would be good sentinel animals.

62.2.5 Phosgene oxime

62.2.5.1 Clinical signs

In both its liquid and vapor forms, phosgene oxime causes severe pain and local tissue destruction upon contact with skin, eyes, and mucous membranes (Sidell et al., 1997). Signs depend on its route of entry, as phosgene oxime exerts its greatest effects in the first capillary bed it encounters.

Dermal, ocular, and respiratory lesions are similar to those caused by mustard gas. Inhalation and oral absorption may cause respiratory tract irritation, dyspnea, and pulmonary edema. Dermal lesions are erythematous and extremely painful. With ocular exposure to phosgene oxime, very low concentrations can cause lacrimation, inflammation, and temporary blindness, while high concentrations can cause permanent corneal lesions and blindness (Sidell et al., 1997; USACHPPM, 2001c). Death is generally due to respiratory arrest.

62.2.5.2 Kinetics

Complete absorption occurs in both dermal and inhalational exposures within seconds (Sidell et al., 1997). Dermal lesions form quickly; grayish tissue damage may be seen within several minutes, and within 1 h, the area becomes edematous. Phosgene will spread in sweat and move to other nonexposed areas of the body (DeRosa et al., 2002). The skin turns brown, and blistering occurs the next day. It takes about 3 weeks for desquamation, necrosis, crust formation, and purulent exudate to occur (Sidell et al., 1997). Pain can last for several days, and healing of dermal lesions can take from 1 month to over a year. Pulmonary edema can be seen on thoracic radiographs within 2 h of high-dose exposure, 4–6 h of moderate exposure, and approximately 8–24 h after low-dose exposure (Sidell et al., 1997).

62.2.5.3 Decontamination and treatment

Animals should be moved into fresh air. Emesis and activated charcoal are not recommended due to the irritant and corrosive effects of phosgene oxime. Dilution with milk or water is recommended for oral ingestions. Sedation and pain control (opioids) may be needed to allow safe decontamination.

Irrigate eyes with tepid water until pH returns to neutral and remains so for 30 min after irrigation is discontinued (Brodovsky et al., 2000). Speed to decontamination after ocular exposure is important since phosgene oxime is absorbed within seconds. Corneal ulcers should be treated with atropine ophthalmics to prevent synechiae formation, and other ophthalmics to aid in reepithelialization (Grant and Schuman, 1993; Brodovsky et al., 2000).

The animal should be bathed with copious amounts of water and a mild soap. Phosgene oxime reacts quickly with tissue, and decontamination is not expected to be entirely effective after pain has been produced. Sodium hypochlorite (0.5%) or isotonic sodium bicarbonate can help neutralize phosgene oxime that has not yet reacted with tissue. Burns should be managed with topical silver sulfadiazine and systemic antibiotics as needed (Roberts, 1988). Horses and sheep should receive tetanus prophylaxis. Other species should be vaccinated on a case-by-case basis.

Monitor oxygenation and thoracic radiographs following significant exposures. Administer oxygen, intubate, and provide assisted ventilation if needed. Nebulized beta-adrenergic agonists can help if bronchospasm develops. Administer i.v. fluids, but monitor for overhydration (Goldfrank et al., 2002).

Phosgene oxime is nonpersistent in the environment, and it hydrolyzes rapidly in aqueous alkaline solutions. Veterinary personnel and rescuers should wear aprons, rubber gloves, and masks when treating patients to avoid secondary contamination.

62.2.5.4 Species susceptibility

There are no controlled studies showing that any species of animal would make a good sentinel for phosgene oxime exposure.

62.2.6 Cyanide and hydrogen cyanide

62.2.6.1 Clinical signs

Cyanide and HCN are classified as blood agents. They inhibit cellular respiration, oxygen utilization, and ATP production, causing deprivation of oxygen to the body at the cellular level (Way et al., 1988). Both arterial and venous blood appears cherry red and unhaired skin may also appear pink due to the accumulation of oxyhemoglobin (Bingham et al., 2001; HSDB, 2008).

Cyanide exposure can cause transient central nervous system (CNS) stimulation, followed by syncope, ataxia, dyspnea, seizures, paralysis, apnea, coma, and death (Hall and Rumack, 1986). The odor of bitter almonds may be noted in gastric or ruminal contents and expired breath. Initial tachypnea is followed by respiratory depression. Chickens develop tachypnea, have rapid eye blinking, hypersalivation, and lethargy (Wiemeyer et al., 1986). Blindness may occur from cyanide-induced damage to the optic nerve and retina (Grant and Schuman, 1993).

Metabolic and lactic acidosis is commonly seen. Death can occur within minutes.

62.2.6.2 Kinetics

Cyanide and HCN can be absorbed by all routes (inhalation, oral, ocular, and dermal). There is rapid diffusion into tissues, and cyanide irreversibly binds to its target sites. Cyanide is metabolized by rhodanese in the liver to thiocyanate and excreted in the urine (Hall and Rumack, 1986). Without administration of an antidote, the half-life for the metabolism of cyanide to thiocyanate is 20 min to 1 h (Feldstein and Klendshoj, 1954).

62.2.6.3 Decontamination and treatment

Remove animals from the affected area. Irrigate eyes for at least 15–20 min with tepid water. Bathe animals thoroughly with soap and water.

Blood gases and serum electrolytes should be monitored (Hall and Rumack, 1986). Blood cyanide levels can confirm exposure, but are not clinically useful. Provide supplemental oxygen with assisted ventilation as indicated. Acidosis (pH $<$ 7.1) should be corrected with i.v. sodium bicarbonate, but may not resolve until after the administration of antidotes (Hall and Rumack, 1986). Benzodiazepines or barbiturates can be used to control seizures.

Cyanide toxicosis progresses so rapidly that treatment is rarely administered to animals in time. If the animal is still alive, but in respiratory distress or a coma, antidotal agents may still be life-saving. The classic treatment for cyanide intoxication includes several steps. Sodium nitrite is given via i.v. over 15–20 min (fast administration causes hypotension). Sodium nitrite reacts with hemoglobin in the RBCs to form methemoglobin. Methemoglobin combines with free cyanide to form cyanomethemoglobin. Sodium thiosulfate is given next and supplies sulfur for the rhodanese reaction (Hall and Rumack, 1987). The sulfur reacts with cyanomethemoglobin to form hydrogen thiocyanate, which is excreted in the urine.

Hydroxocobalamin (Cyanokit) is the newer option and works by combining with cyanide to form cyanocobalamin (vitamin B_{12}) (Hall and Rumack, 1987). Hydroxocobalamin has been shown to reduce mortality in rats, mice, and beagles and has the advantage of producing neither methemoglobinemia nor hypotension, as sodium nitrite does (Hall and Rumack, 1987; Borron et al., 2006).

HCN is lighter than air and has a long half-life in air. However, in open spaces, HCN is rapidly dispersed and is diluted to nontoxic concentrations. Cyanide does not bind to soil or plant material but can mix with water. Contaminated water can be treated with ozone, hydrogen peroxide, or calcium/sodium hypochlorite bleach. Rescue personnel should wear boots, gloves, goggles, full protective clothes, and a self-contained positive-pressure breathing apparatus as the potential for secondary contamination is high (NIOSH, 2005).

62.2.6.4 Species susceptibility

There are significant interspecies differences in the toxicity of HCN (Sousa et al., 2003). Dogs appear to be more susceptible than humans to cyanide poisoning. This is thought to be due to lower levels of endogenous rhodanese (hepatic enzyme that catalyzes the sulfuration of cyanide to thiocyanate) (Aminlari and Gilanpour, 1991). Barcroft (1931) exposed both a man and a dog simultaneously to HCN gas. The 70-kg man and 12-kg dog were exposed to HCN concentrations between 500 and 625 ppm in an airtight chamber. The dog became ataxic at 50 s, unconscious at 74 s, and began to have seizure at 90 s. At 91 s, the man walked out of the exposure chamber with no symptoms, although over the next 10 min, he developed transient nausea and difficulty concentrating (Barcroft, 1931). As HCN is lighter than air, it is probably not that the small dog experienced a higher exposure than the human.

The LDLo (oral) for HCN in humans, dogs, and rabbits is comparable (5.7, 4, and 4 mg/kg, respectively) (Sax and Lewis, 1989). Dogs may be good sentinel animals as they appear to have increased susceptibility relative to humans based on physiological differences. More controlled studies are required, however.

62.2.7 Military nerve agents

62.2.7.1 Clinical signs

Military nerve agents are probably the most toxic of the known CWAs. Military nerve agents are divided into G (for "Germany") agents (such as sarin, soman, and tabun) and V (for "venomous") agents (such as VX). These agents are organophosphates (OPs). Acute exposure to OPs can cause muscarinic, nicotinic, and CNS signs. Muscarinic effects include salivation, lacrimation, urination, dyspnea, diarrhea, and emesis (SLUDDE), along with miosis, bradycardia, hypotension, and bronchoconstriction. Nicotinic effects include muscle fasciculation and weakness (including the diaphragm), tachycardia, hypertension, and mydriasis. CNS effects include restlessness, anxiety, seizures, and coma (Garigan, 1996). VX has CNS effects that are unrelated to AChE activity and prolonged effects may be seen following convulsive doses (Young et al., 1999). Death is due to paralysis of the diaphragm, increased bronchial secretions, or depression of the respiratory center (Garigan, 1996).

While the volatile G agents present a vapor hazard, VX has a high dermal toxicity, even through intact skin, as the liquid does not evaporate quickly (Berkenstadt et al., 1991;

Sidell et al., 1997). A very small drop on the skin may cause sweating and fasciculations at the site. A larger dermal drop may cause loss of consciousness, seizures, apnea, and flaccid paralysis. The toxicity of these substances, listed in descending order on a per-weight basis, is VX, soman, sarin, and tabun (HSDB, 2008). Soman produced severe delayed neuropathy in the atropinized hen assay at 1.5 mg/kg (Willems et al., 1984).

62.2.7.2 Kinetics

Nerve agents can be absorbed by any route (ocular, oral, inhalation, dermal) (HSDB, 2008; RTECS, 2008). The onset of signs and duration of effects depend on the form of nerve gas (vapor, liquid) and the route of exposure. With inhalation, local signs of nasal discharge and respiratory noise begin within 1 min to several minutes and signs can last for a few hours (mild exposure) up to 1–2 days (severe exposure) (Pfaff, 1998). Inhalation of a large amount of the vapor will result in sudden loss of consciousness, apnea, flaccid paralysis, and seizures within seconds to 2–3 min (Sidell et al., 1997). Peak effects are seen within 20–30 min, and death is usually due to respiratory failure (Berkenstadt et al., 1991). Liquid nerve agents applied dermally cause signs starting 3 min to 2 h after exposure. Signs last for 3–5 days.

Ocular exposure to vapor causes miosis, conjunctival hyperemia, and eye pain within 1 min to several minutes. Signs can last 2–3 days. Liquid tabun penetrates the eye quickly and can result in death nearly as rapidly as an inhalational lethal dose (1–10 min) (EPA, 1985b). Ingestion of the liquid causes muscarinic, nicotinic, and CNS signs about 30 min after exposure, the signs can last several hours up to 2–5 days.

Volume of distribution varies little for each of the nerve agents. Sarin is distributed to the brain, liver, kidney, and plasma (Little et al., 1986). Soman and tabun have the highest levels in the hypothalamus (Hoskins et al., 1986; Wolthuis et al., 1986). Soman is unique in that it has apparent storage in body "depots" and is released over time, which can result in eventual death in animals that survive the initial dose (Wolthuis et al., 1986).

Most military nerve agents undergo aging of the OP–enzyme complex, which makes it resistant to therapies (Young et al., 1999). The aging $t_{1/2}$ is only a few minutes for soman, about 5 h for sarin, and greater than 40 h for VX and tabun (Garigan, 1996).

62.2.7.3 Decontamination and treatment

Administer oxygen and remove the animals from the toxic environment. For ocular exposures, flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 min. Wash all animals three times with either soap and water, dilute bleach solution (1:10 with water), ethanol, or a tincture of green soap (Cancio, 1993). Towelettes impregnated with alkaline chloramine and phenol are used by the military (M291 Skin Decontaminating Kit, Rohm and Haas).

Due to the rapid development of signs, emesis is not recommended after oral ingestion. Seizures can be controlled with diazepam or barbiturates as needed. Assisted ventilation may be necessary if signs progress.

Atropine is an antidotal treatment to reverse the muscarinic signs. It blocks the effects of accumulated acetylcholine (ACh) at the synapse and should be continued until the nerve agent is metabolized. Over-atropinization can cause hyperthermia, tachycardia, agitation, mydriasis, and ileus, which can be life-threatening in the horse.

Oximes are used to treat the nicotinic effects (muscular weakness, diaphragmatic weakness, etc.). Pralidoxime (2-PAM) is the oxime of choice in the United States, and is most effective when administered in the first 1–3 h. Due to the quick aging of soman, 2-PAM is rarely given soon enough to be effective (Sidell et al., 1997). 2-PAM can be given up to 48 h after exposure to VX and tabun, due to slow aging (Sidell and Groff, 1974).

AChE activity can be tested in plasma, serum, or whole blood. In most animal species, 80% or more of the total blood AChE activity is in the RBCs, as compared to 50% in humans; therefore, whole blood is the preferred sample for most veterinary diagnostic labs. Inhibition of RBC AChE is interpreted as follows: (1) 10%–20%, no reliable evidence of exposure; (2) 30%–50%, mild poisoning; (3) 50%–70%, medium or moderate; and (4) 70%–90%, severe intoxication. Cholinesterase levels can take weeks to months to return to normal (Grob, 1956; Rengstorff, 1985).

Environmental persistence is estimated to be 0.5–1 day for tabun, 1–2 days for soman, and 5 days for sarin (Garigan, 1996). VX is an oily liquid that remains in the environment for weeks or longer after being dispersed (Garigan, 1996; Sidell et al., 1997; Munro et al., 1999; Budavari, 2000). Contaminated soil should be treated with alkaline substances (such as sodium carbonate, sodium bicarbonate, calcium hydroxide, or calcium carbonate) or chlorine compounds (sodium hypochlorite or calcium hypochlorite) (EPA, 1975).

Protective equipment (rubber gowns, aprons, and gloves), along with respiratory protection, must be worn by rescuers and veterinary personnel. Leather and fabrics absorb OPs and are extremely difficult to decontaminate. Collars, muzzles, and other items should be incinerated. For further details on decontamination of nerve agents, see Chapter 73.

62.2.7.4 Species susceptibility

Nerve agent susceptibility varies widely between species. Animals may be more at risk for greater exposures than

nearby human populations. Both the G and V nerve agents are heavier than air, and animals closer to the ground will be more affected (Rabinowitz et al., 2008). Some species differences can make animals more sensitive than humans, while others will make them more resistant. Rats may be less susceptible than humans, since they possess aliesterases that can reduce the toxicity of certain nerve agents such as soman and tabun (Fonnum and Sterri, 1981; Gupta and Dettbarn, 1987). These enzymes are not present in humans. Nasal breathers, such as rodents, are capable of partially detoxifying nerve agents in the nasal pathways, presumably by hydroxylation and other mechanisms (Garamone, 2003). Carboxylesterase (CarbE) activity in guinea pigs and rabbits may provide protection from soman, when compared to humans (Maxwell et al., 1987).

RBC AChE activity also varies among species. Pigs, sheep, dogs, rabbits, and cats have less RBC cholinesterase activity than humans, which has been associated with increased susceptibility to nerve agents (Leng and Lewalter, 1999; Anonymous, 2000b).

The LCt_{50} (50% lethal concentration via inhalation) for military personnel for different nerve agents has been estimated from animal studies. Humans appear to be more sensitive to tabun than other animals. The LCt_{50} for tabun is 70 mg min/m^3 for humans, 320 mg min/m^3 for dogs, 450 mg min/m^3 for rats, and 960 mg min/m^3 for rabbits (Anonymous, 2000a; NRC, 2003). Goats are more sensitive than humans to VX. The LCt_{50} for goats is 9.2 mg min/m^3, while the human LCt_{50} is 15 mg min/m^3 (Anonymous, 2000a; NRC, 2003).

It is unknown if animals may have decreased dermal absorption compared to humans due to their protective fur or feathers. Comparing dermal exposures in humans and other species also shows differences for the various nerve agents. Mice appear to be more sensitive to dermal sarin than humans; the LD_{50} values for each are 1.08 and 28 mg/kg, respectively (Sidell et al., 1997; RTECS, 2008). The dermal LD_{50} for tabun in mice is 1 mg/kg; for rats, it is 18 mg/kg, and for humans, it is 14 mg/kg. Mice may be slightly more resistant to dermal soman, with LD_{50}s of 7.8 mg/kg for mice and 5 mg/kg for humans.

There are a few cases where shared exposures to nerve agents have been reported for both animals and humans. In 1968, there was an accidental release of two different nerve agents (one is thought to have been VX) in Utah. A flock of sheep that was grazing near the base was noted to be acting "crazy in the head," and thousands died less than 24 h later (Boffey, 1968). Nearby humans, cattle, dogs, and horses did not develop symptoms. The sheep had severely depressed cholinesterase levels, as did the cattle and horses. Cholinesterase testing of dogs and humans was normal. The sheep may have been more affected due to higher exposures through ingestion of contaminated pasture, or by spending more time in the vicinity of the chemical release.

A way that animals could provide an early warning is via their sense of smell. Most animals have much more sensitive olfactory systems than humans. Animals may be able to be trained to sense low nerve gas concentrations (Dalton, 2003).

62.2.8 3-Quinuclidinyl benzilate

62.2.8.1 Clinical signs

3-Quinuclidinyl benzilate (BZ) is a centrally acting synthetic anticholinergic agent. BZ is used as a hallucinogenic and incapacitating CWA. It is about 25 times more potent than atropine and has a very long duration of action. BZ is disseminated as an aerosol, with the primary route of absorption through the respiratory system.

BZ affects both the peripheral nervous system (PNS) and CNS. It inhibits glandular secretions ("dry as a bone"), leading to a dry mouth and foul breath (Holstege, 2006). Cutaneous vasodilation and skin flushing may be noted ("red as a beet") due to decreased capillary tone. Hyperthermia ("hotter than Hades") is due to inhibition of sweating and inability to dissipate heat. Vision loss ("blind as a bat") occurs from a loss of accommodation reflexes, decreased depth of field secondary to ciliary muscle paralysis and mydriasis (Holstege, 2006). Paralytic ileus, which can be fatal to horses, is commonly seen as a result of anticholinergic toxicity (Ketchum and Sidell, 1997; Holstege, 2006).

CNS signs of disorientation, agitation, tremor, ataxia, stupor, coma, and seizures may occur from inhibition of central muscarinic receptors (Ketchum and Sidell, 1997; Holstege, 2006). It is unknown if animals hallucinate like people, but they do appear distressed. Rhabdomyolysis and myoglobinuric renal failure can be seen secondary to seizures and agitation (Holstege, 2006). Sinus tachycardia, hypertension and tachypnea may occur (Ketchum and Sidell, 1997; Holstege, 2006).

62.2.8.2 Kinetics

BZ is lipophilic and easily crosses the blood–brain barrier (BBB), leading to CNS effects. Signs are dependent on the dose and time after exposure. Prolonged effects may occur depending on the dose of BZ absorbed. Tachycardia and dry mouth develop within 15 min to 4 h after exposure, and peak effects occur at 8–10 h (Ketchum and Sidell, 1997). Binding to high-affinity muscarinic acetylcholine receptors (mAChRs) is essentially irreversible for the first 6 h. BZ is excreted via the kidneys (Holstege, 2006). Without treatment following an incapacitating dose, recovery is gradual, requiring 72–96 h (Ketchum and Sidell, 1997).

62.2.8.3 Decontamination and treatment

Move into fresh air and monitor for respiratory distress. Supplemental oxygen and assisted ventilation may be required. Nebulized beta-adrenergic agonists should be used if bronchospasm develops. Due to the method of distribution (aerosol), most exposures are expected to be via inhalation. However, with animals' tendency to groom, dermal exposures can also become oral exposures.

Flush eyes with copious amounts of tepid water for at least 15 min. Animals should be bathed with soap and water. Bathing will not only remove the BZ, but will also provide external cooling to combat hyperthermia. BZ may be detected in urine, serum, or blood, but clinical use is minimal.

Benzodiazepines can be used to control agitation and seizures and prevent rhabdomyolysis.

Treat arrhythmias symptomatically (lidocaine, propranolol, etc.). Physostigmine can be used to treat severe arrhythmias, but it is ineffective if given during the first 4–6 h following the onset of BZ effects.

Monitor electrolytes and renal function tests in symptomatic patients. Administer i.v. fluids to maintain urine output and to protect the kidneys from myoglobinuria. The prognosis is good if animals do not develop rhabdomyolysis or secondary infection. No chronic problems are expected from BZ itself (Holstege, 2006).

BZ is stable and environmentally persistent. "Off-gassing" may occur from contaminated patients. Goggles and masks should be worn by all personnel until the animals are decontaminated (Holstege, 2006). Remove contaminated collars, leashes, harnesses, halters, and other devices, and discard them, as leather and fabrics absorb BZ.

62.2.8.4 Species susceptibility

There are no studies demonstrating that animals are more sensitive than humans.

62.2.9 RCAs (lacrimators)

62.2.9.1 Clinical signs

Chloroacetophenone (i.e., CN, mace, tear gas), chlorobenzylidene malonitrile (i.e., CS, super tear gas), and oleoresin capsicum (OC; pepper spray) are lacrimators used in riot control. They are solid chemicals administered as a fine dust or aerosol spray rather than being true gases. Exposure to lacrimators causes immediate pain, blepharospasm, lacrimation, rhinorrhea, coughing, and sneezing, but usually no permanent tissue damage (Grant and Schuman, 1993; Blain, 2003). With higher ocular concentrations, chemical burns with keratitis and loss of the corneal epithelium may occur (Hoffman, 1967). Oral ingestion can cause eye irritation, lacrimation, vomiting, and diarrhea (Blain, 2003). Laryngospasm may occur due to the irritant effects; it can progress to pulmonary edema, bronchospasm, and bronchopneumonia.

Dermal contact with lacrimators is very painful (Pinkus, 1978). Erythema and blisters are common. The extent of dermal effects depends on the thickness of the stratum corneum, and the extent of exposure (Blain, 2003). High concentrations can cause first- and second-degree burns of the skin (Stein and Kirwan, 1964; Hu et al., 1989). For further details on toxicity of RCA, see Chapter 12, Blister agents.

62.2.9.2 Kinetics

The effects of lacrimators occur very quickly. Pain, salivation, coughing, rhinorrhea, sneezing, and erythema begin within seconds of exposure and can last approximately for an hour (Blain, 2003). Laryngospasm, bronchospasm, and pulmonary edema may occur up to 48 h (usually 12–24 h) postexposure (Folb and Talmud, 1989). Gastrointestinal signs resolve over 24 h (Solomon et al., 2003). Erythema disappears over 48 h and coughing may persist for weeks after exposure (Blain, 2003). One of the CS metabolites is cyanide, but cyanide toxicosis does not appear to occur (Cucinell et al., 1971).

62.2.9.3 Decontamination and treatment

Animals may need to be sedated to be able to treat them safely. Move animals into fresh air and monitor for respiratory distress. Supplemental oxygen may be needed. Laryngospasm may require intubation to permit adequate ventilation. Inhaled beta-2 agonists (i.e., albuterol and salbutamol), corticosteroids, and aminophylline may help reduce bronchospasm (Folb and Talmud, 1989). Thoracic radiographs should be monitored if pulmonary edema is expected (Stein and Kirwan, 1964).

Flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 min. Diphoterine solution can be used for decontamination of both eyes and skin after exposure to lacrimators (Viala et al., 2005). Ocular signs resolve approximately 3–7 min after diphoterine application. Animals should be bathed with soap and copious amounts of cold water. Using small amounts of water can actually increase irritation (Lee et al., 1984). Topically applied magnesium hydroxide–aluminum hydroxide–simethicone suspension (Maalox Max) caused resolution of signs within 2 min of application following exposure to OC. If chemical burns develop, clean wounds with a mild, disinfectant soap and water. Pain control and antibiotics may be needed. Tetanus toxoid should be given if burns are present. With oral ingestions, antacids may help decrease gastrointestinal signs.

Secondary contamination is common and personnel should wear aprons, rubber gloves, and masks as needed. Contaminated items can be washed in cold water (hot

water will cause residual gas to vaporize) with soap or allow nonwashable items to air out for a few days. Most lacrimators dissipate quickly, but CS may be micronized and mixed with an antiagglomerant agent (CS1), which remains active for up to 5 days. A similar formulation mixed with silicone (CS2) remains in the environment for up to 45 days (Hu et al., 1989).

62.2.9.4 Species susceptibility

There are no controlled studies showing that animals are more sensitive to lacrimators than humans.

62.2.10 Ricin and abrin (toxalbumins)

62.2.10.1 Clinical signs

Ricin and abrin are toxalbumins, which are plant lectins with a specific affinity for animal cell receptors. Ricin is from *Ricinus communis* (castor bean plant, castor oil plant, mole bean). The castor bean plant is grown throughout the United States as an ornamental. Most animals are exposed to ricin by eating the seeds, but ricin has been used as a CWA. Abrin is found in the *Abrus precatorius* plant (Buddhist rosary bead, crab's eyes, Indian bead, jequirity bean, rosary pea).

Variable toxicity is seen in cases of oral ingestion. Seeds swallowed whole may not cause any problems at all (Kinamore et al., 1980). The amount of toxalbumins in the seeds will vary by size, weight, moisture content, region, season, and the period of plant growth at the time of harvesting. The ricin content of castor beans can vary from 1% to 10% (Balint, 1974). The abrin content in *A. precatorius* seeds is estimated at 0.15% (Lin et al., 1971). Toxicity and death have occurred with ingestions of one to two chewed beans of either plant. Ricin levels can be measured in plasma and urine but are not clinically useful (Kopferschmitt et al., 1983).

Vomiting, abdominal pain, and bloody diarrhea are the most common signs seen after ingestion (Kopferschmitt et al., 1983). Toxalbumins cause severe gastrointestinal lesions of the oropharynx, esophagus, and stomach. The lesions are clinically similar to alkaline burns. Fluid losses can lead to dehydration, electrolyte disturbances, hypotension, and tachycardia (Ingle et al., 1966).

Liver damage occurs in toxalbumin toxicosis, resulting in elevated liver values. Glycogen stores decrease, gastrointestinal absorption of glucose decreases, and hypoglycemia occurs (Lampe, 1976). Mild to moderate CNS depression is commonly observed. Seizures are reported more frequently in animals than in people (Hart, 1963).

Ricin is among the most toxic compounds known when given parenterally. Parenteral toxicity is much greater than oral toxicity. The oral lethal dose of ricin is estimated to be 1 mg/kg (Kopferschmitt et al., 1983). When given by injection, the lethal dose of ricin drops to about 1 μg/kg (Budavari, 2000). With an inhalation exposure, signs seen are cough, dyspnea, arthralgias, fever, and death (Griffiths et al., 2007). Other organ system dysfunctions may not occur. For more details on toxicity of ricin and abrin, see Chapter 28. For other toxic chemicals as potential chemical warfare agents, see Chapter 27, and for toxicokinetics and toxicodynamics of DFP, see Chapter 53.

62.2.10.2 Kinetics

Gastrointestinal effects usually develop in under 6 h (Kopferschmitt et al., 1983). The cytotoxic effects on the liver, CNS, kidney, and adrenal glands may not occur for 2–5 days postexposure. With an inhalation exposure, symptoms begin within 8 h. Death occurs about 48 h after a parenteral or oral exposure (Budavari, 2000).

Glycoproteins are poorly absorbed from the gastrointestinal tract. Many cell surfaces contain receptors specific for ricin and the toxin is taken up by active transport. The primary site of distribution of ricin is the liver, spleen, and adrenal cortex in the mouse. A high concentration was also found in the bone marrow (Godal et al., 1984). In rats, abrin is distributed primarily to the liver (12%) and kidney (3%) (Lin et al., 1971).

The metabolism and elimination of toxalbumins is poorly understood. Ricin is eliminated by first-order kinetics when injected i.v. into mice and human cancer patients (Godal et al., 1984). The plasma half-life in humans is about 2 days (Kopferschmitt et al., 1983).

62.2.10.3 Decontamination and treatment

There are no specific treatments for toxalbumin exposure. Aggressive decontamination is recommended. With ingestions, emesis should be induced in asymptomatic species that can vomit (e.g., dogs, cats, ferrets, and swine). Activated charcoal can bind orally ingested toxalbumins. Monitor asymptomatic animals for 8 h for dehydration, electrolyte disturbances, elevated liver enzymes, and hypoglycemia. Intravenous fluids are very important to maintain normovolemia and urine output. Caprine, ovine, and murine antiricin antibodies have been tested but are not clinically available (Lemley and Wright, 1991; Wannemacher et al., 1991; Whitfield et al., 2017).

If the toxalbumin was distributed by air, move the animal into fresh air and monitor for respiratory distress. If cough or dyspnea develops, administer supplemental oxygen. Wash animals thoroughly with soap and water. Eyes should be flushed with copious amounts of tepid 0.9% saline or water for at least 15 min. Other treatments are the same as for an oral exposure.

62.2.10.4 Species susceptibility

There are no controlled studies demonstrating that animals are more susceptible to developing acute effects than humans.

62.3 Concluding remarks and future directions

The lack of controlled studies comparing animals and humans needs to be addressed if animals are to be used as sentinels. The existing studies and anecdotal reports do not provide enough convincing evidence (Rabinowitz et al., 2008). Sentinels would need to demonstrate easily recognizable signs before the emergence of human illness. A good sentinel could have either greater susceptibility to a particular toxin relative to humans or a shorter latency time from exposure to onset of signs (Cottrell and Morgan, 2003).

With any suspicious outbreaks, the local health department, poison center, law enforcement agencies, and the US Federal Bureau of Investigation (FBI) should be contacted immediately. Due to the absence of a nationwide surveillance system for animal diseases, the responsibility for detecting possible outbreaks of unusual symptoms in animals will fall to several different groups of people. Farmers, agriculture officials, veterinarians, animal control officers, wildlife rehabilitators, (animal) poison control centers, and the lay public (such as animal owners) may all be involved in detecting outbreaks.

During a chemical warfare incident, the human casualties should be addressed first, followed by the animal casualties. Situations in which herds of livestock or flocks of poultry are affected are going to be much more complex to manage than exposures to house pets. The simple logistics in getting the personnel and equipment to the site of exposure can be daunting, if not impossible. Handling livestock can be dangerous, especially for untrained personnel. The use of antidotes and other pharmaceuticals must be documented in food-producing animals and withdrawal times for meat and milk must be followed. In many herd situations, humane euthanasia may be the best solution.

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Chapter 63

Threats to wildlife by chemical and warfare agents

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63.1 Introduction

Wildlife, in its natural habitat, has an economic value inclusive of the experience of viewing, amateur and commercial photography, research, and the preservation of genetic diversity and natural ecosystems (Sangpikul, 2017). In many countries, wildlife-based ecotourism and other related commercial enterprises are important contributors to the gross domestic product. Ecotourism is a growing industry, especially if a country has unique or rare wildlife species and ecosystems. Wildlife has traditional value in aboriginal cultures as a source of food, as materials for garments, footwear, and shelter, religious ceremonies, and is essential for aboriginal people to live in synchrony with nature. Some countries are investing in the intensive management of wildlife and wildlife habitats to sustain wildlife as a renewable resource (Child et al., 2019). Sport and trophy hunting of wildlife is a source of income for governments in licensing fees and taxes from employment of local people as guides and from taxation of accommodation and travel infrastructure. Worldwide, governments are under pressure to protect and ensure wildlife survival, inclusive of the ecosystems necessary for wildlife to feed, breed, and thrive. Protection of wildlife resources relies on governments enacting and enforcing a variety of laws and regulations (Sellar, 2009; Linacre, 2009; Maisels et al., 2013). A form of wildlife-ecological terrorism has been used to deprive self-sustaining aboriginal people of the necessities of life provided by the ecosystem for their independent existence. Wildlife poaching is a criminal activity generally focused on protected predators, damage-causing herbivores and predators, and poaching animals for economic gain from the sale of animal-source protein and illegally obtaining body parts for monetary gain (Sellar, 2009; Cooper and Cooper, 2013; Maisels et al., 2013). Wildlife have a similar sensitivity to the toxic effects of physical and chemical agents as domestic animals. Toxic chemicals have been and can be used as terrorist weapons to target wildlife.

Terrorism is a systematic use of violence to create fear and economic deprivation to achieve political and other objectives (Stankov, 2018). Terrorist activities are generally performed to destabilize society and undermine governmental control, create economic hardship, and deprive targeted human populations (Gleick, 2006). Grudge is emerging as a motivator for committing terrorism and is a reason humans react to unwanted actions of animals (Stankov, 2018; Ntemiri et al., 2018). Terrorists are opportunistic, and target that have long-term impacts are attractive (UN-CTED, 2017). Counterterrorism is difficult in rugged terrains and in prime wildlife habitat areas. By using the ecosystem that supports wildlife as their operational platform, terrorists can avoid detection by antiterrorism technology and trained personnel that are used in populated areas. Wildlife terrorist and commercial poachers use “guerilla-type” hit-and-run tactics (Desphande, 2009). Wildlife can be a direct or indirect target for terrorist attacks, and terrorist activities against wildlife can be undertaken to disrupt or destroy regional income (Felbab-Brown, 2018). Terrorist attacks against governmental and commercial operations can occur in more remote areas, and such attacks could have an immense impact on wildlife, including fish and other aquatic organisms. In some countries, military belligerents undertake hit, run, and hide warfare, and bushmeat (meat from wildlife) is an important part of their rations. Wildlife can be

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used in counterterrorism efforts as sentinels of chemical agents released by terrorists. The anomalous behavior of sentinel species equipped with tracking devices can be used to monitor the movement of belligerents (Rabinowitz et al., 2008; Sahin and Ercan, 2008).

There have been reports of wildlife suffering direct and indirect damage during armed conflicts, causing food shortages and disruption to the local economies (Brown, 1999; de Merode et al., 2007; Hanson et al., 2009; Nellemann et al., 2014; Felbab-Brown, 2015; Hanson, 2018). Some authors describe the environmental impact of armament races and wars as the treadmill of environmental destruction (Clark and Jorgenson, 2012). Hostile activities and terrorism are interconnected. Wildlife and their habitats can suffer as collateral damage from air and other attacks by belligerents and also military actions undertaken to reduce hiding places for belligerents and actions taken to reduce the bushmeat that these belligerents rely on for food (Olson and Morton, 2019). In addition, illegal trade in wildlife parts can be an important source of revenue to support belligerent activities (Beyers et al., 2011; Felbab-Brown, 2015, 2018). Corrupt government officials can also profiteer from the sale of wildlife parts. People displaced by terrorism and other forms of conflict may be forced to depend on bushmeat for survival.

Wildlife, especially scavengers, can destroy forensic evidence. The evidence required in the prosecution of poachers and wildlife terrorism can be destroyed by scavenging wildlife. Wildlife itself can be an impediment to forensic investigations, for example homicide, by destroying or otherwise altering evidence (Linacre, 2009; Sincerbox and DiGangi, 2018). For example, birds and rodents scavenging can impact the recovery of biological evidence during forensic investigations (Chipman et al., 2004). Counterterrorism devices can fortuitously impact procedures to reestablish endangered wildlife. For example, X-ray devices used in counterterrorism and used to detect smuggling in wildlife parts can damage semen and embryos from endangered species being shipped to other locations to ensure heterogenicity (Gloor et al., 2006).

63.2 Infrastructure and potential widespread chemical contamination

The resiliency of an ecosystem to human depredation is important when considering activities undertaken in human conflict (Orians and Pfeiffer, 1970; Banout et al., 2014). The effects of chemicals used to control an ecosystem during conflicts can have long-term multigenerational consequences (Frey, 2013). Dams holding water impoundments are considered a terrorist target (UN-CTED, 2018). Uncontrolled release of water from impoundments has large-scale impacts on humans and all terrestrial and aquatic animals living in the drainage basin. The reasons for impounding freshwater are generally multifaceted, including water for domestic, agricultural, and industrial uses, and energy storage for hydroelectrical generation. Mine and other industrial wastewaters are stored by impounding in tailing ponds (waste slurry storage facilities) (Rico et al., 2008; UNEP, 2017). These liquefied wastes contain toxic leftovers after the valuable elements and other components are removed from ores, slurries, and/or process water. These impoundments can contain antimony, arsenic, cadmium, copper, lead uranium, zinc, process chemicals including cyanide, fine mineral particulates, etc. (Hudson-Edwards, 2016). These impoundments can contain an immense amount of heavier-than-water waste materials and dams holding these impoundments of mining waste have failed, resulting in the sudden release of kinetic energy and toxic materials resulting in large-scale disasters (Bowker and Chambers, 2016). Dams holding back liquefied waste, due to their lack of best-practice construction, can be inherently unstable (Bowker and Chambers, 2016; Schoenberger, 2016; UNEP, 2017). Terrorist and military destruction of dams weaponizes the kinetic energy and toxic materials contained by these dams. Water impoundments can be weaponized by clandestine methods that cause failure of dams (Nett and Ruttinger, 2016). At this time, no reports of large-scale releases of toxic water linked to terrorism or warfare have been found. Examples are given to illustrate the scale of these disasters because of their enormous potential for environmental impact, and the destruction of human life and health. The failure of the Fundao tailings dam on November 5, 2015, is historically the worst release of toxic water (60 million m^3) in Brazil (Marta-Almeida et al., 2016). There were 18 confirmed deaths and one person missing. For 17 days, the released tailings traveled through the Rico Doce River basin before reaching the Rio Doce estuary in the Eastern Brazilian Marine Ecoregion of the Atlantic. In some regions the riparian deposits of tailing mud were 40 cm thick. Studies have estimated the tailings dispersed for hundreds of kilometers in the Atlantic estuary and impacted marine biota (Marta-Almeida et al., 2016; Gomes et al., 2017). In Italy, breach of the Val di Stava dam released an estimated 180,000 m^3 of liquid tailings into Stava and caused 268 deaths. The contents were discharged into the Aviso River and caused extensive environmental damage. The failure of the Mount Polly dam in Cariboo Region of British Columbia (Canada) released approximately 25 million m^3 of liquified tailings into Polley Lake and subsequently along Hazeltine Creek channel into the west basin of Quesnel Lake (Petticrew et al., 2015; Byrne et al., 2018; Hatam et al., 2019). It is estimated that the tailing pond materials will have a long-term effect on the biota in Quesnel Lake and present a potential hazard to aquatic food webs and

the growth and survival of important fish species and the biogeochemical cycle. Aquatic species are used by aboriginal peoples living in this area. The release of 50–100 tonnes of cyanide from a tailings pond into the Sasar River, and eventually reaching the Danube River, had a devastating impact on aquatic wildlife in Eastern Europe (Cunningham, 2005). Recently, the tailing pond dam at the Vale Mine in Brazil collapsed causing loss of human life and large long-lasting environmental impacts. One can only imagine the public and political reaction that would have occurred if this incident was claimed to be an act of terrorism.

63.3 Pyroterrorism and wildlife

Pyroterrorism can cause collateral damage to wildlife, or wildlife and habitat can be a direct terrorist target. Pyroterrorism is the use of an incendiary attack (weaponizing fire) to achieve the objectives of the terrorist, and pyroweapons are used in military conflicts (Baird, 2006; Marsden et al., 2014). A well thought through pyroterrorism plan can be easily and inexpensively accomplished, be a huge media event, and compared to other targets, offers low risk of being apprehended (Marsden et al., 2014; Desphande, 2009). Pyroterrorism, in terms of igniting wildfires (bushfires), generally has built-in media coverage of the inferno. Terrorist-linked and/or anarchist-linked wildfires are considered to have occurred in Spain, Estonia, Greece, Turkey, and Israel, but links of wildfires to terrorists are likely underinvestigated (Marsden et al., 2014; Desphande, 2009). Wildfires can have huge economic loses, including the destruction of personal and environmental assets, habitat, the cost of firefighting, the loss of human life, and negative impacts on surface and ground water (Baird, 2006; Desphande, 2009; Rodrigues et al., 2019). Wildfires have also been shown to have impacts on wildlife habitats and wildlife dynamics (Nimmo et al., 2018). Fish and other aquatic organisms, and animals in the food chain, can be impacted by runoff water, including the runoff of water after its use in firefighting (de Vries et al., 2017; Robinne et al., 2018). Rain water, which is atmospheric wash water, can contain pollution emitted during wildfires and be deposited long distances from the fire site. Wildfires remove vegetation, generally increase water runoff after rainfall events and thereby increase toxic substances and sediment entering aquatic systems (Brito et al., 2017; Nunes et al., 2017; Jensen et al., 2017; Hallema et al., 2018). Lands quarantined because of contamination with radioactive compounds are considered targets for pyroterrorism. The radionuclides at the contaminated site become incorporated in the biota and are released in the products of combustion (Evangeliou et al., 2014). Building fires also have environmental impacts (Noiton, 2001). The most visible chemical release during a fire is smoke, less visible are the thousands of cubic meters of water used to extinguish a building fire (Scholz, 2014). Extinguishing water that is not vaporized (fire-water) can run off the fire site and contaminate surface water (Holemann, 1994). Fire-water can also contaminate ground water, the fire-water runoff contains products of combustion, chemicals stored in the building when the fire occurred, and chemicals used in firefighting. The polluted fire-water can cause major environmental impacts in the receiving waters that last for years and also result in the need for expensive remediation (Flowles, 2001; Giger, 2009). Runoff water from even small structural fires can present a threat to aquatic ecosystems (Noiton, 2001). In summary, chemicals released by wildfires and individual building fires can have significant short- and long-term effects on the health of wildlife and the ecosystem.

63.4 Candidate chemical agents

63.4.1 Background

Chemical agents inclusive of pesticides have a history of being used for various reasons to kill wildlife (Ogada, 2014). Chemical agents have also been used to poison water sources to prevent use by insurgents (Purkitt and Burgess, 2002). Wildlife and humans can share the same surface water; in this case, it is unknown if wildlife will be affected. Pesticides are used to control damage-causing wildlife and are used in poaching. There are worldwide concerns regarding illicit pesticides being used for clandestine activities including terrorism and their impact on non-target wildlife (Whitlow et al., 2005; UNICRI., 2016; Patocka et al., 2018). Under regulations, certain pesticides, because of their toxicity, have restricted use and some governments have banned them. Pesticides that are obsolete and no longer manufactured or prohibited in one country may legitimately be manufactured and sold in another country, making them available for smuggling activities (Shakarjian et al., 2016). Compounds that are highly toxic when ingested orally are considered candidate chemicals for weaponization and are especially dangerous to wildlife because residual levels in carcasses can cause relay toxicity in scavenging and opportunistic feeders.

63.4.1.1 Tetramethylenedisulfotetramine

63.4.1.1.1 Background

Tetramethylenedisulfotetramine (2,6-dithia-1,3,5,7-tetraazatricyclo[3.3.1.13,7]-decane,2,2,6,6-tetraox- ide, tetramine, TETS, TMDT, CAS No. 80-12-6) has historical use as a one-dose pesticide, but is reemerging as an illicit synthetic neurotoxic chemical and is a potential chemical warfare agent (Barrueto et al., 2003; Whitlow et al., 2005;

Shakarjian et al., 2016; Patocka et al., 2018). TETS accounts for many human deaths due to accidental and forensic exposures (Wang et al., 2016). It is an odorless, tasteless, white, crystalline powder with a solubility in water of 250 mg/L [ToxNet. https://chem.nlm.nih.gov/chemidplus/rn/80-12-6 (accessed March 2019)], and it can be used in water and food baits. It is estimated that 50% of the rodenticides in China contain TETS, where the Chinese name is "Du Shu Qiang" (Patocka et al., 2018; Owens et al., 2009). TETS is considered to be 100 times more toxic than cyanide and a lethal dose for humans is 7–10 mg and it is considered to be one of the most dangerous toxic substances that can be used in food and water baits (Whitlow et al., 2005). TETS can be used as a lethal seed coating and is translocated to plants in sufficient quantities for the plant to be fatal to rodents and lagomorphs (Spencer et al., 1954; Shakarjian et al., 2016). TETS persists in soil and lethal levels can be translocated to plants for up to 4 years. TETS is also used in fabrics as a stiffening and antifungal agent (Patocka et al., 2018). TETS has been identified in accidental and homicidal poisonings (Shakarjian et al., 2016).

63.4.1.1.2 Mechanism of action

TETS has a short latency between oral ingestion and onset of clinical signs. It selectively and irreversibly binds with the neuronal γ-aminobutyric acid A receptor ($GABA_A$) and disrupts physiological regulation of the chloride channel (Zhao et al., 2014; Shakarjian et al., 2016). The net toxicological effect is dysregulation of membrane regulation of chloride and subsequent depression of neuronal inhibition in the central nervous system (CNS). TETS is a noncompetitive inhibitor of the $GABA_A$ receptor and, compared to insects, has a 300–5000-fold selective affinity for the mammalian $GABA_A$ receptor. Inhibitory activity in the CNS is decreased and clinical manifestations include seizures, with onset occurring 20–30 minutes after TETS ingestion. The toxicity of TETS is generally considered to be more potent than sodium monofluoroacetate. The oral LD_{50} of TETS for most species is 0.1–0.3 mg/kg of body weight. Rabbits dosed with TETS at 0.4 mg/kg body weight and killed 1 hour later had detectable levels (0.07–0.238 μg/g) in the liver, kidney, heart, and lung (Xiang et al., 2001). TETS can be identified in liver after being fixed in formalin for 4 months (Xiang et al., 2001). TETS is excreted in urine, which can be used for forensic investigations (Zeng et al., 2006). Scavenging animals and birds can be poisoned with TETS because of the stability of TETS in tissues and body fluids. There appear to be sex and age differences in sensitivity to TETS with females and younger animals likely being more sensitive than mature males (Laukova et al., 2018).

63.4.1.1.3 Pathology and detection

The pathology of TETS in humans has been reported (Zhang et al., 2011). Pathological observations in poisoned humans were edema of the brain, hemorrhages in the brain stem, and myocardial degeneration in the papillary muscles. Cardiac myopathies of varying distribution are a common finding in the majority of species. A reasonable assumption is that similar pathology and levels in tissues and urine would occur in other species. Human tissues and urine can be assayed for TETS (Xiang et al., 2001; Zeng et al., 2006). Analytical methods for TETS include liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry and immunoassay (Owens et al., 2009; Vasylieva et al., 2017).

63.4.1.2 Sodium monofluoroacetate and sodium fluoroacetamide

63.4.1.2.1 Background

Sodium monofluoroacetate (SMFA; compound 1080) and sodium fluoroacetamide (SFA, compound 1081) are candidate chemicals for terrorists and criminal activities targeting humans, domestic animals, and wildlife (Holstege et al., 2007). Mechanistically, the toxicology of SFA is the same as SMFA (Wang et al., 2016). SMFA is a white powder that has the appearance of flour or sugar and is stable in storage over a long period of time and is heat-stable to 200°C. It is soluble in water but insoluble in ethanol and lipids. In water solution, SMFA is degradable by microorganisms. Both SMFA and SFA were/are used as single-exposure pesticides and, because of their toxicity, have been banned or placed on licensed restricted use by many governments. One use is control or elimination of nonindigenous problem wildlife. As an interesting comment, SMFA and closely related compounds are the poisonous principal in several species of plants growing in South America, Africa, and Australia (Lee et al., 2014). The use of SMFA as a poison to eliminate unwanted animals and birds is restricted in many countries, primarily because of large kills in nontarget species (Ataria et al., 2000). Countries with nonindigenous mammal populations use SMFA to control non-native animal species that disrupt and endanger indigenous wildlife (Potter et al., 2006; Allen et al., 2014). The water solubility of SMFA means that it can easily be added to water, baits, and feedstuffs and is generally accepted for consumption by target and nontarget species. The use of SMFA can be limited to use in devices that target problem predators. For example, SMFA in some jurisdictions has been limited to neck collars containing multiple SMFA-filled chambers that are ruptured when the predator attempts a fatal attack on the neck (Burns and Connolly, 1995). SMFA is also considered to have the potential for weaponization.

In 2014, an incident occurred in New Zealand wherein the terrorist or blackmailers alleged to have contaminated milk products as a protest against the government approval of SMFA to control non-native fauna (Cooney et al., 2016). No milk was found to actually contain SMFA. There are recent reports of SMFA-linked livestock and pet poisonings and the forensic issues were not clearly identified (Giannitti et al., 2013; Brower et al., 2017; Adaska et al., 2018). These reports also point out the resources and manpower required to establish a diagnosis when the etiology is a restricted toxic substance.

63.4.1.2.2 Toxicology of sodium monofluoroacetate

SMFA is absorbed from the gastrointestinal tract, respiratory tract, mucous membranes, and wounds (Holstege et al., 2007). Different routes of exposure do not have a remarkable effect on toxicity. The mechanism of action for SMFA is blockage of the mitochondrial tricarboxylic acid cycle. The toxication reaction is fluoroacetate being converted to fluoroacetyl-CoA and subsequent conversion to fluorocitrate by citrate synthase. The enzyme aconitase catalyzes the conversion of citrate to isocitrate and is inhibited by fluorocitrate. Inactivation of aconitase blocks the tricarboxylic acid cycle. The heart and CNS are simultaneous targets for SMFA. Animals poisoned by SMFA are a hazard to scavengers, opportunists, and carnivores. The sensitivity of species to SMFA is given in Table 63.1.

TABLE 63.1 Estimated lethal dose of SMFA in selected animals.

Mammal or bird	Lethal dose (mg/kg body wt)
Dog	0.1–0.2
Coyote and fox	0.1–0.3
Cattle	0.2–0.7
Pig	0.3–0.4
Cat	0.3–0.5
Horse	0.5–1.8
Human	0.5–5.0
Goat	0.5–0.7
Magpie	0.6–2
Rabbit	0.8
Chicken	6–18
Duck	7–9

Source: Data from Robinson, W.H., 1979. Acute toxicity of sodium monofluoroacetate to cattle. J. Wildl. Manage. 34, 647–648; Ataria, J. M., Wickstron, M., Arthur, D., et al., 2000. Biochemical and histopathological changes induced by sodium monofluoroacetate (1080) in mallard ducks. NZ Plant Protect. 53, 293–298; Goh, C.S., Hodgson, D.R., Fearnside, S.M., et al., 2005. Sodium monofluoroacetate (Compound 1080) poisoning in dogs. Aust. Vet. J. 83, 474–479 (Goh et al., 2005); Collicchio-Zuanaze, R.C., Sakate, M., Langrafe, L., et al., 2010. Hematological and biochemical profiles and histopathological evaluation of experimental intoxication by sodium fluoroacetate in cats. Hum. Exp. Toxicol. 29, 903–913 (Collicchio-Zuanaze et al., 2010).

63.4.1.2.3 Clinical signs of intoxication

Clinical signs of SMFA toxicity are due to the effects on the CNS and heart. Herbivores can show more cardiac signs as compared to carnivores. The sequence of cardiac events is arrhythmias, tachycardia, inefficient pumping (weak pulse), and death when the heart starts to fibrillate. Clinical signs observed include ataxia, collapse, and a short interval of agonal struggling and seizures (Robinson, 1979). Carnivores can have neurological signs of hyperesthesia, aimless wandering, frenzied running, vocalization, incoordination, emesis, opisthotonus, coma, and death. Thermoregulation may also be disrupted. Death occurs anywhere from 1 to 24 hours after ingestion of SMFA. Poisoned birds can "fall" from the sky and die soon after hitting the ground or show nervous system signs and an inability to fly. Relay (secondary) intoxication by SMFA and its metabolites can occur. An animal that has ingested SMFA and vomits can be lethally intoxicated, and the animal ingesting the vomit can also be lethally intoxicated (Brower et al., 2017). Animals poisoned by SMFA can repetitively be lethal to animals and birds that consume them by relay poisoning.

63.4.1.2.4 Pathology and forensic chemistry

Pathological findings in SMFA poisoning are those of acute heart failure and hypoxia. Focal to diffuse degenerative to necrotic lesions occur in the myocardium. Inflammatory exudates may be observed. Skeletal muscular lesions were observed in mallard ducks experimentally poisoned with SMFA (Ataria et al., 2000). Tissues and body fluids can be assayed for SMFA and its metabolites by gas or liquid chromatography methods (Giannitti et al., 2013; Cooney et al., 2016; Adaska et al., 2018).

63.4.1.3 Cyanide

63.4.1.3.1 Background

Cyanide (CN^-) is poisonous to essentially all animal species and is a listed chemical for potential weaponization by terrorists. Cyanide, in different chemical formulas, can be admixed in a variety of baits, including water. Cyanide is also used in the extraction of gold and silver ores and presents environmental risks (Donato et al., 2007; Griffiths et al., 2014). Catastrophic release of a large volume of tailing pond slurry containing high concentrations of cyanide and heavy metals into river systems has

occurred but this event was not linked to terrorism (UNEP, 2000; Macklin et al., 2003; Cunningham, 2005). The rupture in January 2000 of a Romanian dam holding 100,000 m^3 of cyanide-contaminated slurry water caused an environmental disaster (Macklin et al., 2003; Cunningham, 2005). An estimated 50–100 tonnes of cyanide and heavy metals were released into the Sasar River and then flowed through the Lapus River to the Somes River, then to the Tisza River, and finally emptied into the Danube River. The breach in the dam was attributed to a number of factors, including a sequence of rainfall occurrences. Scientific evaluations showed this event caused severe mortalities in the aquatic system. It is feasible that terrorist or criminal activities could target dams holding tailings ponds and cause a huge sudden release of contaminated water, creating a down-river environmental disaster that targets drinking water safety, wildlife, tourism, and international relations. Cyanide also has a history of being used to control unwanted wildlife and is toxic in the marine environment (Wiemeyer et al., 1986). Vehicles transporting cyanide for mining and other uses could be a target of terrorists and used to release cyanide into flowing water (Kravitz and Blair, 2019).

63.4.1.3.2 Toxicology

The pathophysiology of acute cyanide poisoning is due to its rapid diffusion into tissues and impairment of oxidative phosphorylation and other biochemical pathways (Beasley and Glass, 1998). The primary enzyme target is cytochrome oxidase by the high affinity of CN^- for ferric iron in the oxidized enzyme. Oxygen utilization is inhibited, and lactate accumulation rapidly causes metabolic acidosis. Clinical signs of cyanide intoxication in birds have been described (Wiemeyer et al., 1986). The progression of clinical signs of cyanide poisoning in vultures is ataxia, eye blinking, head bowing, wing droop, increased ataxia, seizures with tail fanning, opisthotonus, gasping, and death. The progression of clinical signs in kestrels, owls, and quail is reported to be more violent. Exemplary lethal dose 50 (LD_{50}) of cyanide is given in Table 63.2. Time from ingestion to death is dose-dependent. Terrorist and criminal actions that release cyanide into the environment, especially into aquatic ecosystems, can kill large numbers of wild animal species. Release of cyanide-polluted water can have long-term consequences on a freshwater ecosystem (Lakatos et al., 2003). Cyanide poisoning of wildlife can also be collateral damage from arson-type terrorist activities due to its presence in runoff water from firefighting and in runoff from burnt-over lands (Barber et al., 2003). Freshwater fish are highly sensitive to free cyanide and fish mortality can occur at concentrations of less than 20 μg CN^-/L of water (Barber et al., 2003; Eisler and Wiemeyer, 2004). The lethal level of cyanide in rainbow trout is changed by water temperature and exercise (McGeachy and Leduc, 1988). Young fish are generally more sensitive to the adverse effects of cyanide on thyroid hormone homeostasis (Brown et al., 2004). Exposure to cyanide can reduce the number of viable eggs produced by sexually maturing rainbow trout (Lesniak and Ruby, 1982). Cyanide can be used in poaching to stun, kill, and harvest fish and its use can have long-term consequences on the aquatic ecosystem (Authman et al., 2013).

TABLE 63.2 Estimated LD_{50} of cyanide in wildlife.

Bird or animal	LD_{50} (mg/kg body wt)
Black vulture	4.8
American kestrel	4
Eastern screech owl	8.6
European starling	17
Chicken	21
Japanese quail	10
Coyote	4.1

Source: Data from Sterner, R.T., 1979. Effects of sodium cyanide and diphacinone in coyotes (Canis latrans): Applications as predacides in livestock toxic collars. Bull. Environ. Contam. Toxicol. 23, 211–217 (Sterner, 1979); Wiemeyer, S.N., Hill, E.F., Carpenter, J.W., et al., 1986. Acute oral toxicity of sodium cyanide in birds. J. Wildl. Dis. 22, 538–546.

63.5 Selected pesticides

63.5.1 Background

Pesticides and neurotoxic chemical warfare agents could be used in acts of terrorism against a targeted wildlife population, or wildlife can be killed as collateral damage. There is a societal movement to restrict the use of and/or ban highly toxic pesticides (European Parliament, 2014). The reasoning for these restrictions and/or banning these pesticides is multifactorial, including small volume required for single-dose killing, relay poisoning, demonstrated forensic uses, and the potential for weaponization. Restricted and/or banned pesticides can be smuggled from a country of use, and illegally sold or made available for use (UNICRI., 2016). Pesticides, including restricted and banned substances, are used forensically to kill wildlife (Ogada, 2014; Botha et al., 2015; Ntemiri et al., 2018). Birds and other wildlife, particularly scavengers, are poisoned by consuming the carcasses of intentionally poisoned animals including birds. Granular pesticides, likely mistaken for food, can be voluntarily ingested by some bird species. Vertebrate deaths have been reported from forensic and off-label use of insecticides (Botha et al., 2015). Mathematical models can be used to

estimate the probability of bird deaths resulting from field application of pesticides (Mineau, 2002). Birds, mammals, fish, and other aquatic organisms can be nontarget species from pesticides that enter water systems from surface water. Highly lethal pesticides are used in poaching and could be used by terrorists to target wildlife. The toxicology of pesticides in birds has recently been reviewed (Sobhakumari et al., 2018).

63.5.2 Incidents of intoxication

Waterways and flood areas can be contaminated with pesticides, especially insecticides, and result in bird deaths (Hunt et al., 1995; Elliott et al., 2008). Granules of carbofuran in flooded fields have been incriminated as the cause of duck deaths and the cause of death in raptors scavenging dead or debilitated ducks. Seeds treated with insecticides to control insect damage can be toxic to birds (Millot et al., 2017). A "lone wolf" could use treated seeds to poison seed-eating birds. Bats in a county administration building were poisoned with dichlorodiphenyltrichloroethane (DDT) (Buchweitz et al., 2018). The source of the banned DDT and the perpetrator were not identified, and the most likely route of exposure was dermal. Some people kill bats because of a psychological reaction to scenes depicted in horror movies (chiroptophobia) and fear of zoonotic diseases caused by bat-borne Lyssavirus.

63.6 Castor bean (*Ricinus communis*)

63.6.1 Background

The castor bean and its poisonous principal ricin have been identified as having potential for weaponization. Ricin is a Category B terrorism agent and Schedule 1 chemical warfare agent. Annually, 400,000−610,00 tons of castor beans are grown and crushed to produce castor oil, with ricin remaining in the meal (cake) byproduct (Patel et al., 2016). Various preparations of *Ricinus communis* also are used in traditional medicine (Marwat et al., 2017). There are inconclusive reports of Canada geese being killed due to castor bean ingestion. In Texas, castor bean intoxication could have been the cause of deaths of 10,000 ducks in 1967, and 2000 ducks in 1969−70 (Jensen and Allen, 1981). The death of 1673 ducks occurred again in Texas in 1971. A castor bean was found in the gastrointestinal tract of one duck during pathological examination and castor beans had been grown in the area. Although inconclusive, this report does incriminate voluntary ingestion of castor beans as the cause of death in ducks. Sheep and dogs poisoned by voluntary ingestion of castor beans have been reported (Roels et al., 2010). A potential terrorist activity could be making castor beans available to wildlife.

63.6.2 Toxicology and pathology

Ricin, a toxalbumin that inactivates ribosomes, is the toxic principal in *R. communis*. It is in the seeds and the meal byproduct. Ricin, after a series of steps of being taken into the cell and activated, damages the 28S rRNA and thereby inhibits protein synthesis (Grela et al., 2019). One molecule of ricin in the cytosol can be fatal to a cell. Ducks taken from the area where 2000 ducks had died in 1969 through 1970 were examined by diagnostic procedures (Jensen and Allen, 1981). The toxins of *Clostridium botulinum* and other pathogenic bacteria were not identified. Catarrhal enteritis was identified with hemorrhage into the intestine. Seed parts suggestive of *R. communis* were found in scrapings of the wall of the proventriculus. Fatty degeneration of hepatocytes was also observed. In a study in which ducks were force-fed whole castor beans (Jensen and Allen, 1981), progressive signs of acute intoxication were passage of blood-streaked mucus, leg paresis, loss of mobility of the wings, sitting, prone recumbency, and death. Necropsy findings were whole and fragments of castor beans and congestion of the liver. Histopathology changes included severe fatty degeneration of hepatocytes, granulocytic infiltration into portal areas, pulmonary congestion, and peribranchial hemorrhage, and necrosis and hemorrhage were observed in the spleen. These findings support the field observation that some waterfowl may consume castor beans that can result in fatal intoxication. For comparison, the reported histopathology in dogs after consumption of castor beans resulting in fatalities are, for the kidney, renal tubular degeneration and necrosis in a multifocal arrangement (Roels et al., 2010). Glomerular lesions are eosinophilic fluid-appearing material and unidentified debris surrounding the glomerular capillaries. Fibrin was not observed, and the focal thickening of the Bowman's capsular membrane was characteristic of a membranous glomerulonephritis. Lesions in the jejunum were congestion and hemorrhage in the mucosa, focal enterocyte necrosis occurring in the villus crypts, erosion and disruption of villi, infiltration by degenerative leukocytes, and abundant nuclear debris. Focal areas of hepatic degeneration were observed and the heart lesion was myocardial degeneration. Splenic and lymph node follicles had pyknotic and karyorrhectic nuclei. In sheep ingesting castor beans, there was hyperemia and hemorrhage in the abomasum and intestinal tract (Aslani et al., 2007). Histopathology showed hemorrhage, coagulation necrosis, and mononuclear infiltrate in the abomasum, and ulcerative fibrinous enteritis. The liver showed hepatocellular degeneration and centrilobular necrosis. Histopathology in the heart was both subepicardial and subendocardial hemorrhage with multifocal coagulative necrosis of the myocardium. The kidneys showed acute tubular necrosis and medullary

hemorrhage. The observation that wild ducks voluntarily ingest castor beans provides evidence that there is risk that terrorists could successfully use ricin against wild ducks and other wild waterfowl species.

63.6.3 Water baits

Water baits can be used by terrorists to deliver toxic substances to target wildlife. Water-soluble toxic substances are used in water baits to control animal pests. Generally, these substances are nonirritating, odorless, tasteless, and highly toxic. The substances used in water baits are substances that kill by single lethal dose. Toxic substances that can be weaponized for use to poison water supplies are of concern to military planners. For wildlife, a small nonflowing water source is used, and substances to enhance attraction can be added to the water. Sodium nitrite in a micronized coating is being tested to control wild pigs (wild boars) populations (Snow et al., 2018). Sodium nitrate is also water-soluble, is a contaminate of ground water, and could be used in water baits. The nitrite reacts with iron (ferrous) in hemoglobin to form methemoglobin (oxidized ferric form). Methemoglobin has very low oxygen-carrying capacity and causes hypoxia, loss of consciousness and death. The solubility of sodium nitrite is 820 g/L of water at 20°C. Sodium azide has been used as a terrorist weapon and can be added to water.

63.7 Concluding remarks and future directions

Wildlife as a direct or indirect target generally has not been considered in the emergency management of terrorist events using chemical agents. Wildlife, because of economic value and emotional attachment, can be a direct target, and because they can be important sources of food and income for some regions and countries. Disruption of revenues from ecotourism could be an objective for terrorists. Wildlife can also be collateral damage from terrorist attacks and acts of war. Government restrictions and bans on pesticides and other toxic substances do not reduce their use against wildlife targets (Chiari et al., 2017). Regardless of the cause, wildlife can be intoxicated when chemicals and physical agents are released into the environment. Emergency planning needs to include terrorist attacks on wildlife, especially in areas where wildlife is concentrated during breeding and migration, and where wildlife congregates during the nonbreeding season. Hopefully, the shooting of mountain gorillas, an act of ecoterrorism to disrupt the Virunga National Park, is not an indication that these events will become more common (Jenkins, 2008; Actman, 2018). There is a need to develop better models for investigating and understanding the total impact of all causes of incidents involving wildlife because these incidents have much in common with the damage caused by terrorism and warfare (Etterson, 2013). The methods and procedures for forensic investigations of wildlife deaths need continual development along with diagnostic infrastructure (Linacre, 2009).

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Chapter 64

Pharmacological prophylaxis against nerve agent poisoning: experimental studies and practical implications

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64.1 Introduction

Inhibitors of cholinesterases are very important chemicals in the group of organophosphates (OPs). These compounds are used in industry, in veterinary and human medicine, and unfortunately in military [chemical warfare agents (CWAs)/nerve agents] and terrorist activities (Japanese incidents in 1994−95). The bulk of these compounds is used as pesticides (insecticides, acaricides, etc.). According to the World Health Organization (WHO), more than 1 million serious accidental and 2 million suicidal poisonings with insecticides occur worldwide every year, and of these approximately 200,000 deaths occur, mostly in developing countries (Eyer, 2003; Bajgar et al., 2004, 2007a). The mechanism of action, prophylaxis, and treatment of intoxications with OPs are currently a very timely topic.

The term *prophylaxis* is sometimes unclear. Generally, it is limited to medical countermeasures applied just before penetration of a toxic agent into the organism without further antidotal therapy. It is also described as a pretreatment, but usually antidotal treatment is performed. Thus, when treatment is unnecessary, it can be described as prophylaxis. Although successful prophylaxis can be observed for some OPs, full protection of the organism without postexposure treatment, especially for soman poisoning, remains open. When treatment is given after the exposure, the term *posttreatment* is used. It is obvious that when the drug is administered prior to nerve agent exposure with the aim of protecting the organism, exposure to these agents is expected, and therefore postexposure therapy can be used; that is, *pretreatment* could be considered the right term. The term *prophylaxis* used in this chapter is limited to medical countermeasures applied shortly before penetration of a toxic agent into the organism with the aim of protecting the organism against the toxic agent.

Prophylaxis against nerve agents has been described and summarized in various publications (Bajgar, 2009, 2012; Bajgar et al., 2004, 2007a, 2013; Bajgar and Klement, 2011; Layish et al., 2005; Patocka et al., 2006; Tuorinsky, 2008; Elsonghorst et al., 2013; Kuca et al., 2013). The basic mechanism of action of OPs is based on their ability to inhibit the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) at cholinergic peripheral and central synapses (Marrs et al., 1996; Bajgar et al., 2004; Masson, 2011; Colovic et al., 2013). In general, prophylaxis can be focused on protection of AChE against inhibition using reversible cholinesterase inhibitors. The level of OP can be diminished by using enzymes that hydrolyze these agents or enzymes that bind the agents (to specific proteins or to antibodies) and thereby reduce the OP level and inhibition of cholinesterases—AChE and butyrylcholinesterase (BuChE) (EC 3.1.1.8; the scavenger effect) in the organism; this can be described as detoxification. Another approach to prophylaxis is based on using present antidotes and other drugs (Bajgar et al., 2004, 2007a, 2009, 2012, 2013; Bajgar and Klement, 2011; Kuca et al., 2013). Different drugs or their combinations can also be used. A simple schema describing the fate of the agent in the organism and possible target sites for prophylaxis is shown in Fig. 64.1.

In the schema, basic reactions for the effect of nerve agents are indicated—they are penetration of the agent

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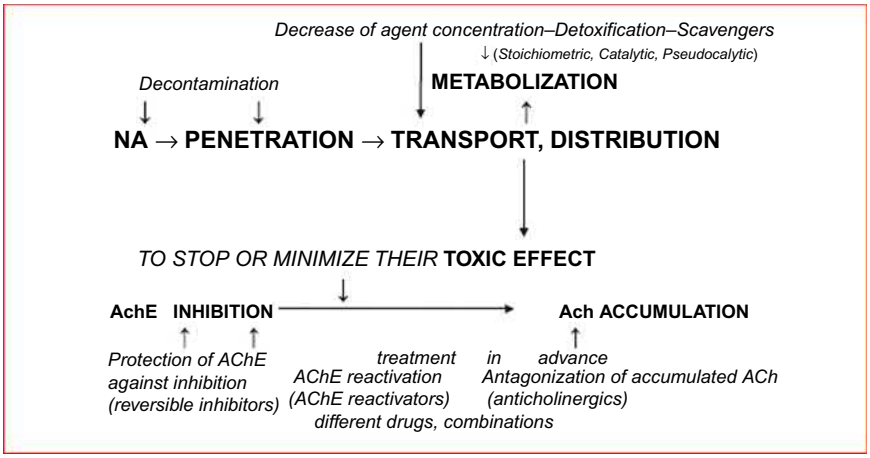

FIGURE 64.1 Four basic reactions of OP in the organism (in capitals) and possible approaches for prophylaxis (in italics). *Modified from Bajgar, J., 2004. Organophosphates nerve agent poisoning: mechanism of action, diagnosis, prophylaxis and treatment. Adv. Clin. Chem. 38, 151–216; Bajgar, J., 2009. Development of antidotes against nerve agents in the Czech Republic. ASA Newslett. 09-06 (135), 7–9; and Gupta, R.C., 2009. Handbook of toxicology of chemical warfare agents. In: Gupta, R.C. (Ed.), Handbook of Toxicology of Chemical Warfare Agents. Academic Press/Elsevier, Amsterdam, p. 1147.*

into the organism through different ways of administration; after penetration into the blood (transport system), the agent is distributed to the places of metabolic and toxic effects. To stop or minimize the toxic effect, different possibilities can be explored. Because inhibition of AChE is the main trigger and basic pharmacodynamic factor, protection against AChE inhibition is one possible process using reversible AChE inhibitors. Treatment in advance (administration of anticholinergics and reactivators) is another possibility, and the use of different drugs minimizing the toxic action is the third possible approach for prophylaxis. Certainly, all these principles can be combined.

64.2 Protection of acetylcholinesterase against inhibition

Intact AChE is a basic requirement for normal function of the organism, and thus for effective prophylaxis. The enzyme is changed in a way that will make it resistant to OP. This can be achieved by using reversible inhibitors, preferably carbonates (CMs), which are able to inhibit AChE reversibly, and after spontaneous recovery of the activity (decarbamylation) normal AChE serves as a source of an active enzyme.

The ability of some CMs to protect an organism poisoned with an OP has been known for many years (Koelle, 1946; Koster, 1946). Physostigmine and neostigmine have been used to protect animals against DFP. The number of OPs studied for protection has been expanded, as well as the number of CMs studied. The results are very dependent on experimental conditions; nevertheless, the protective effects of physostigmine, aminostigmine, pyridostigmine, and others against AChE inhibition caused by different OPs (mostly soman) have been demonstrated (Patocka, 1989; Marrs et al., 1996; Tonkopii, 2003). It has been described that weak inhibitors are able to protect cholinesterases from strong inhibitors (paraoxon; Petroianu et al., 2005a, 2007). Non-OP inhibitors were also tested (Kassa et al., 2012; Lorke et al., 2012, 2013; Petroianu et al., 2013). It appeared from many studies that the cholinesterase inhibitor pyridostigmine was the most promising prophylactic drug, particularly against soman poisoning (Gordon et al., 1978, 2005; Patocka et al., 1991; Anderson et al., 1992; Koplovitz et al., 1992; Maxwell et al., 1993; Bajgar et al., 1994; Kassa and Bajgar, 1996; Kassa and Fusek, 1998, 2000; Tuovinen, 1998; Koupilova and Kassa, 1999; Kassa et al., 2001a,b). On the basis of these results, pyridostigmine was introduced into some military units as a prophylactic drug against nerve agents. Its prophylactic effect (like the effects of other CMs) is increased with its dose; however, with a higher dose, the side effects are also increased. This problem can be solved by adding pyridostigmine-antagonizing drugs, anticholinergics.

Many anticholinergics have been tested for their ability to protect the organism against intoxication with soman (and other nerve agents). On the basis of this research, the prophylactic combination PANPAL, composed of pyridostigmine with trihexyphenidyl and benactyzine (Bajgar et al., 1994, 1996; Kassa and Bajgar, 1996; Kassa and Fusek, 1998, 2000; Fusek et al., 2000, 2006; Kassa et al., 2001a), was introduced into the Czech army, and later into the Slovak army. The presence of anticholinergics allowed for an increase in the pyridostigmine dose and, simultaneously, an increase its prophylactic efficacy (Kassa et al., 1997; Bajgar et al., 2004, 2007a; Fusek et al., 2006). Moreover, prophylaxis with PANPAL improves following postexposure antidotal therapy with anticholinergics and oximes (Bajgar et al., 2004; Kassa, 2006). The prophylactic antidotal combination called PANPAL has no side effects, as demonstrated on volunteers: no statistically significant different changes in the actual psychic state, as well as no negative changes in the dysfunction time, were observed. An improvement of the tapping test following PANPAL administration was

demonstrated. A decrease in the heart frequency 60 min following PANPAL administration lasting 480 min and returning to normal values within 24 h was demonstrated (Fusek et al., 2006).

Other CMs also have a good prophylactic efficacy, especially physostigmine and aminostigmine (due to their central effect which is contrary to pyridostigmine; Tuovinen and Hanninen, 1999; Kim et al., 2002; Tonkopii, 2003). Experimental studies with transdermal administration of physostigmine suggest a serious interest in the prophylactic use of this drug (Levy et al., 1992; Jenner et al., 1995; Walter et al., 1995; Kim et al., 2005; Cho, 2012; Cho et al., 2012). A similar approach was described with scopolamine (Che et al., 2011).

Structurally different inhibitors from the CM and OP groups were also studied. From these compounds (preferably binding to the AChE anionic site), tacrine, its 7-methoxyderivative (7-MEOTA) and huperzine A, were considered and experimentally studied with respect to prophylaxis in vitro and in vivo (Bajgar, 2004). The most interesting results were obtained with huperzine A (Lallement et al., 2002; Gordon et al., 2005; Bajgar et al., 2007b; Karasova et al., 2009; Wang et al., 2013).

64.3 Scavengers

Keeping AChE intact can be achieved by eliminating the agent before its penetration into the target sites containing the enzyme. In this case, the detoxification principle can be used in two different ways: administration of enzymes splitting the OP or specific enzymes (cholinesterases) that bind the OP. The enzymes that hydrolyze nerve agents are called *catalytic* scavengers, and the enzymes that bind nerve agents are described as *stoichiometric* scavengers. OPs are bound to the exogenously administered enzyme or decomposed by the enzyme and thus the nerve agent level in the organism is decreased (the action of a "scavenger"). Enzymes, which hydrolyze OPs, were thoroughly studied by Raveh et al. (1992) and Li et al. (1995). Catalytic scavengers are enzymes displaying a turnover with OP nerve agents as substrates, allowing rapid and efficient protection (Masson et al., 1998; Ditargiani et al., 2010; Gupta et al., 2011; Kuca et al., 2013; Otto et al., 2013). Paraoxonase and similar enzymes are prospective hydrolyzing scavengers (Aharoni et al., 2004; Rochu et al., 2008; Valiyaveettil et al., 2010, 2011a,b; Trovasler-Leroy et al., 2011). On the other hand, many studies have been completed using cholinesterases as scavengers. AChE was used as a prophylaxis against OP poisoning, and a correlation has been found between protection and the blood−enzyme level in mice (Wolfe et al., 1987; Raveh et al., 1989). BuChE and AChE were observed to be very effective in protection against OP intoxication (Ashani et al., 1991; Doctor et al., 1991, 1997, 2002; Maxwell et al., 1993, 1998; Moore, 1996; Saxena et al., 1997; Bird et al., 2010). The idea to use the plasma as a source of cholinesterase to eliminate nerve agent action after peritoneal dialysis with human plasma in rats was also described (Bajgar et al., 1982), but without further development.

The administration of enzymes as scavengers seems to be very promising: the enzyme is acting at the very beginning of the toxic action, without interaction with the target tissues and without side effects (Doctor et al., 1997, 2002; Saxena et al., 2004, 2011; Huang et al., 2007). All of these features are of great interest, and they are yielding practical results—namely, isolation of the enzyme, examination for lack of an autoimmune response, and establishment of pharmacokinetic and pharmacodynamic properties (Saxena et al., 2002). Recombinant human BuChE can be produced from the milk of transgenic goats (Cerasoli et al., 2005) commercially produced as Protexia. Moreover, BuChE pretreatment also showed protective effects on AChE inhibition in the brain regions following low-level sarin inhalation exposure: the enzyme (BuChE) administered into the bloodstream does not influence erythrocyte AChE, plasma BuChE activity is increased, and the brain AChE is not affected. Following intoxication with a nerve agent, the agent is bound to plasma BuChE (now existing in excess), and proportionally bound to erythrocyte AChE; both enzymes are inhibited, however, to a lesser extent in comparison with the unpretreated situation. The part of the nerve agent bound to both enzymes represented a loss of the agent, and the diminished level of nerve agent is able to penetrate the brain. Therefore, AChE activity in the brain is protected (Sevelova et al., 2004; Bajgar et al., 2007c).

The efficacy of cholinesterases as a bioscavenger of OP can be enhanced by combining enzyme pretreatment with oxime reactivation, since the scavenging capacity extends beyond a stoichiometric ratio of cholinesterases to OP. Human BuChE has previously been shown to protect mice, rats, and monkeys against multiple lethal toxic doses of OP anticholinesterases (anti-AChEs; Maxwell, 1992; Allon et al., 1998). An interesting approach can be simultaneous administration of BuChE and reactivators; BuChE acts as a scavenger binding the nerve agents. A reactivator acting as a *pseudocatalytic* bioscavenger reactivates BuChE simultaneously, and reactivated enzyme serves as a new scavenger (Jun et al., 2007; Kovarik et al., 2010).

This approach was tested using human BuChE (Jun et al., 2011). The best reactivators were trimedoxime and obidoxime, but only in the case of OP insecticides (paraoxon, methamidophos, and leptophos; Jun et al., 2007, 2011; Musilova et al., 2009). Thus, currently available reactivators cannot be of interest and practical use as pseudocatalytic scavengers for nerve agents.

Given our increasing knowledge of bioengineering and biotechnology, recombinant human BuChE with a good protective effect against nerve agents was obtained from transgenic animals. A connection between these two enzymes (binding and hydrolyzing) is possible, with the aim of obtaining a modified enzyme splitting OPs and simultaneously reacting with AChE as a scavenger (Broomfield et al., 1997). Antibodies against OPs are at the research stage and are more focused on the detection of OPs (Lenz et al., 1997; Miller and Lenz, 2001).

Current progress in prophylaxis against nerve agents is summarized in the review of Bajgar et al. (2019).

64.4 Prophylaxis with current antidotes

The aim of this approach was to achieve a sufficient level of antidotes in the blood circulation before intoxication, so the antidotes can be tested as prophylactics. This can be characterized as "treatment in advance." The problem with their use, however, is the timing, duration, and achievement of sufficient levels of these antidotes after administration. The antidotes currently used for the treatment of OP poisoning in this context include anticholinergics, reactivators, and anticonvulsants (Bajgar et al., 2004, 2007a). The prophylactic efficacy of antidotes (as demonstrated in treatment studies) is good; administration of the antidotes typically takes place very shortly (i.e., within minutes) after intoxication. Another study showed good prophylactic efficacy of pyridostigmine or ranitidine with pralidoxime against paraoxon poisoning (Petroianu et al., 2005b, 2006). Extension of the duration of the effects of the antidote by achieving sufficient levels in the blood by oral administration is not possible (especially reactivators, as they are decomposed in the gastrointestinal tract) and therefore is excluded. Consequently, other routes of administration were sought. Transdermal administration of one of the most effective reactivators (HI-6) was shown to be the most realistic approach (Dolezal et al., 1988; Bajgar, 1991). The final result was a new prophylactic transdermal antidote TRANSANT, containing the patch impregnated with HI-6. This preparation was clinically tested (including dermal sensitivity) without any harmful effects; field testing was also successful, and TRANSANT was introduced into the Czech army and later into the Slovak army (Bajgar et al., 2004; Fusek et al., 2007). If its administration is combined with PANPAL, the two anticholinergics contained in PANPAL are able to prevent and treat symptoms of nerve agent poisoning. Therefore, a combination of TRANSANT and PANPAL administered as a pretreatment provides the best prophylactic efficacy compared to pyridostigmine or PANPAL administered alone (Bajgar et al., 2004). The anticonvulsant benzodiazepine drugs (e.g., diazepam, midazolam, alprazolam, triazolam, and clonazepam) were studied, but isolated prophylactic administration did not work to very good effect (Herink et al., 1990, 1991; Kubova et al., 1990).

64.5 Prophylactic use of other drugs

Prophylactic administration of different drugs (alone or in combination) against intoxication with OPs was studied. Calcium antagonists (e.g., nimodipine), neuromuscular blockers (e.g., tubocurarine), adamantanes (e.g., memantine), and the opiate antagonist meptazinol (Galli and Mazri, 1988; Gupta and Dettbarn, 1992; McLean et al., 1992; Marrs et al., 1996; Karlsson et al., 1998; Stojiljkovic et al., 1998) were also tested, offering different results with limited practical utility. On the other hand, a positive prophylactic effect has been demonstrated with procyclidine (e.g., antimuscarinic, antinicotinic, and the anti-NMDA receptor drug; Myhrer et al., 2002, 2003), metoclopramide (Hasan et al., 2004), clonidine (Loke et al., 2001), or procyclidine and donepezil (Haug et al., 2007). The prophylactic effect of a group of drugs with anticholinergic and/or antiglutamatergic properties (e.g., benactyzine, biperiden, caramiphen, procyclidine, and trihexyphenidyl), with respect to their anticonvulsant properties, was studied to prevent damage of the central nervous system induced by seizures. Only procyclidine and caramiphen antagonized soman-induced seizures (Myhrer et al., 2008a; Schultz et al., 2014). Among the different drugs tested, procyclidine appears to be an effective anticonvulsant, with few cognitive side effects (Myhrer et al., 2008b). Procyclidine with physostigmine (administered transdermally) showed very good prophylactic efficacy against soman in dogs; moreover, this efficacy was increased by using antidotal therapy with HI-6 and atropine (Kim et al., 2005). Special importance can be placed on suramine (a protease inhibitor). Administration of this compound prior to soman intoxication (and followed by administration of atropine) showed good prophylactic effects (Cowan et al., 1996). However, all these studies are experimental, and they have not reached the practical output stage. The combinations of various drugs as prophylactics can be of very different character. They can be used simultaneously (a combination of different drugs) or as a pretreatment and a posttreatment with different antidotes. Administration of pyridostigmine (or other AChE inhibitors) prior to intoxication and treatment with different drugs is a typical example (Anderson et al., 1992, 1997; Kassa, 1995; Bajgar et al., 1996; Kassa and Bajgar, 1996; Tuovinen and Hanninen, 1999; Kim et al., 2002). Aerosolized scopolamine was described to protect guinea pigs against inhalation toxicity to sarin (Che et al., 2011). There are other combinations as well, such as the administration of triesterase (Tuovinen and Hanninen, 1999; Tuovinen et al., 1999), procyclidine

TABLE 64.1 **Drugs used in the prophylaxis against OP nerve agent poisoning (relatively promising drugs are in bold).**

Principle	Drug group	Drug	Duration	Equipment of the army	Efficacy	Comment
Protection of cholinesterase against inhibition	Carbamates	**Pyridostigmine, Aminostigmine, Physostigmine**	8 h	PYRIDOSTIGMINE BROMIDE	+++	Dose limited, side effects. Alone is not very effective, following antidotal treatment enhances its effect
		Syntostigmine, Eptastigmine, Mobam				
		Decarbofuran, Heptylphysostigmine				
	Others	**Huperzine A**				
		Tacrine, Methoxytacrine				
	Organophosphates					
		TEPP, Paraoxon				
		Ethyl-4-nitrophenylphosphonate				
	Aminophenols	Eseroline				
Simulation of treatment	Anticholinergics	**Biperidene**, **Scopolamine**, **Benactyzine** Atropine, Aprophen, Hyoscine				Transdermal administrations of scopolamine, physostigmine and HI-6 were studied
		Adiphenine, Caramiphen				
		Pentamethonium, Mecamylamine				
		Trihexyphenidyl				
	Reactivators	HI-6				
		PAM, Obidoxime, Trimedoxime, Methoxime	8 h	TRANSANT (HI-6, transdermal administration)	+	Alone is not effective
	Others	**Suramine** Benzodiazepines, Tubocurarine Memantine, Procyclidine Nimodipin, Clonidine				
Detoxification	Cholinesterases enzymes hydrolyzing OP monoclonal antibodies against OP	**Butyrylcholinesterase, Mutants**				Very perspective PROTEXIA
		Acetylcholinesterase				
		Triesterase Paraoxonase				
Combinations			8 h	PANPAL (pyridostigmine, trihexyphenidyle, benactyzine)	++++	Efficacy is increased with following antidotal treatment
				PANPAL + TRANSANT	+++++	In combination, the best prophylactic efficacy

(Kim et al., 2002; Myhrer et al., 2002, 2003), clonidine (Loke et al., 2001), or sustained release of physostigmine and scopolamine (Meshulam et al., 2001). The results depend on experimental conditions, but this approach (administration of different drugs) has yielded some good results. However, up to now, they have been only on an experimental level.

Clearly, there is a wide range of prophylactics. The drugs used in prophylactics against nerve agents are summarized in Table 64.1. Some of them (bold in the table) can be studied further. Only three prophylactics have been introduced by armies—pyridostigmine alone; PANPAL, composed of pyridostigmine, benactyzine, and trihexyphenidyl; and TRANSANT (dermal administration of HI-6). Another prophylactic drug, Protexia, can be considered as well. The efficacy of different prophylactics against nerve agents expressed as prophylactic indexes is shown in Fig. 64.2.

Current prophylactics have a few problems. Reversible cholinesterase inhibitors have side effects caused by their parasympathomimetic activity. This issue can be addressed by adding anticholinergic drugs. Isolated administration of anticholinergics also can be helpful prophylactically; however, this effect can be supported by adding reversible inhibitors. The main problem with bioscavengers is immunoreaction, which can be solved by modifying the scavenger. Using the approach of the treatment in advance, reactivators are used, but the main issue is their route of administration. Further, conducting additional research into improving prophylaxis is difficult.

A comparison of the features of different prophylactics is summarized in Table 64.2. Various criteria are used, including cost, side effects, and difficulty of use. The best combination of effects, cost, and accessibility is observed when PANPAL and TRANSANT are used together. These prophylactics are shown in Fig. 64.3.

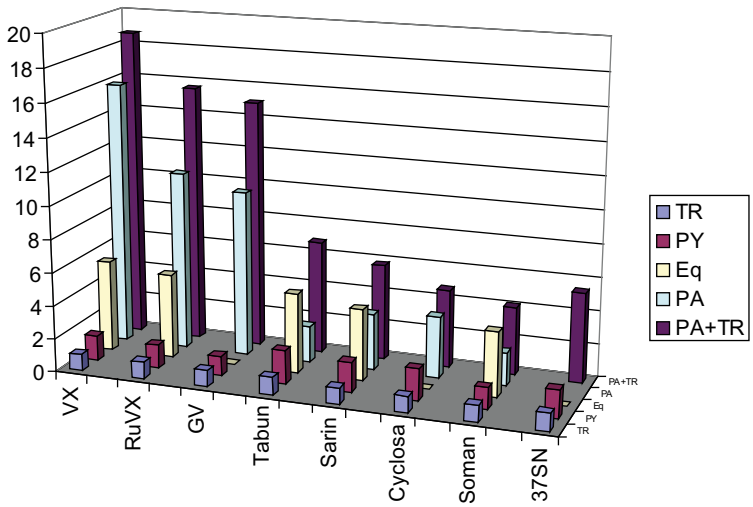

FIGURE 64.2 Efficacy (expressed as a prophylactic index) of different prophylactics (TR: TRANSANT; PY: pyridostigmine; Eq: equine butyrylcholinesterase; PA: PANPAL; PA + TR: combination of PANPAL and TRANSANT) against selected nerve agents in rats. The results are means only. Prophylactic effect of equine butyrylcholinesterase was not tested for 2-(dimethylamino)ethyl *N,N*-dimethylphosphoramidofluoridate (GV), cyclosarin, and 37SN. *Elaborated using data from Bajgar, J., 2009. Development of antidotes against nerve agents in the Czech Republic. ASA Newslett. 09-06 (135), 7–9; Bajgar, J., Klement, C., 2011. Chemical warfare agents misuseable in the civilian sector, history and misusing of CWA (in Czech). In: Klement, C. (Ed.), Extraordinary Events in the Public Health Service (in Slovak). PRO, Banska Bystrica, pp. 206–257; Bajgar, J., 2012. Nerve Agents Poisoning and Its Treatment in Schematic Figures and Tables. Elsevier, Waltham, MA.*

TABLE 64.2 Comparison of various prophylactics using different criteria of their evaluation.

Criterion	Pyridostigmine	PANPAL	Protexia	TRANSANT
Introduced in other armies	Yes	No	No	No
Introduced in the Czech army	No	Yes	No	Yes
Accessibility	++++	++	++	++
Cost	+	++	++++	+++
Difficulty to use	+	+	++(i.v., i.m.)	++(self-help)
Prophylactic effect	+	+++	+++	Ineffective alone
Side effects in recommended dose	No	No	Antigenic properties	No
Duration of prophylaxis	6–8 h	6–8 h	5–8 h	8 h
Increase of effect of followed antidotal treatment	+	++	No	++

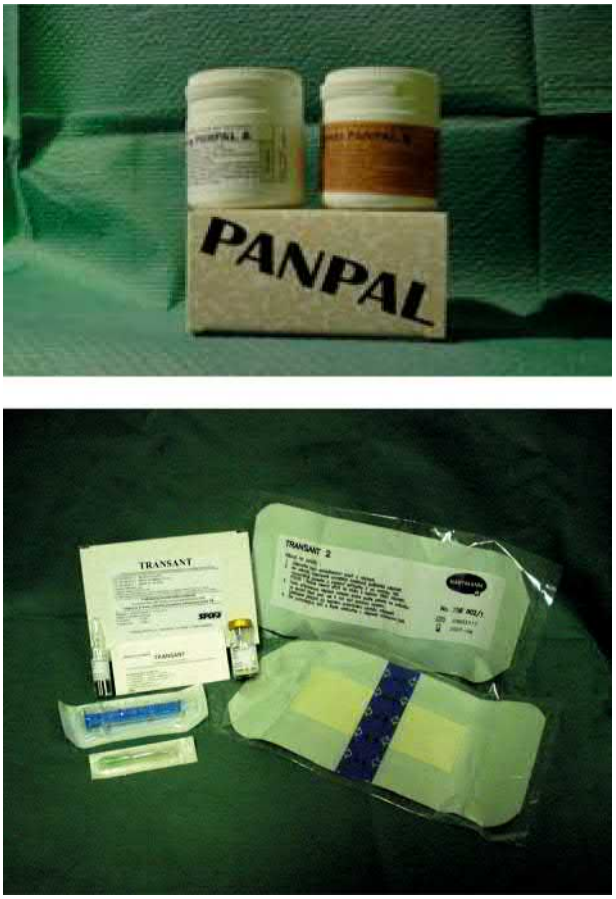

FIGURE 64.3 Two prophylactics: PANPAL (top) and TRANSANT (bottom).

It appears from these results that simple prophylaxis (without postexposure treatment) against OPs is insufficient. Therefore, pyridostigmine is important as a prophylactic drug, especially when it is combined with postexposure antidotal treatment. For further development, it is necessary to search for novel prophylactic drugs and new routes of administration. In this context, preparations of cholinesterases are of special importance for the development of more effective prophylactics.

64.6 Concluding remarks and future directions

There are many drugs being tested for their prophylactic efficacy against nerve agent intoxication. However, only three of these (pyridostigmine alone, PANPAL, and TRANSANT) have been introduced into military practice. The prospective approach for the future seems to be to search for more effective drugs or their combinations using modern techniques (e.g., molecular modeling), including new routes of administration, and the use of purified enzymes, especially cholinesterases and paraoxonases produced by biotechnology.

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Chapter 65

Prophylactic and therapeutic measures in nerve agents poisonings

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65.1 Introduction

Ever since the so-called G-agents (NATO code, denoting their German origin)—tabun (GA), sarin (GB), and soman (GD) were synthesized by Gerhard Schrader and his co-workers at IG Farben Industrie in Nazi Germany in the late 1930s and early 1940s—antidotes against these extremely toxic organophosphorus compounds (OPCs) started to be developed. The V-agents ("V" taken from the word "venomous"), like *O*-ethyl-*S*-(diisopropylamino)-ethyl methyl phosphonothioate (VX), were discovered by a British scientist Dr. Ranajit Ghosh in 1952 and later added to the US armamentarium (Stojiljković, 2019).

Despite some specificities and differences, all these compounds, called nerve agents, have the same very distinct mechanism of action: they irreversibly inhibit the acetylcholinesterase (AChE) in all human tissues and, depending on the gravity of intoxication, pose a serious threat to human survival. The resulting accumulation of acetylcholine (ACh) in the cholinergic synapses causes overstimulation of central and peripheral muscarinic and nicotinic receptors. On the clinical level, these biochemical effects manifest themselves as acute cholinergic syndrome, that occurs in the case of intoxication with OPC or carbamates, with the only difference being in the fact that the carbamate-induced AChE inhibition is reversible and therefore self-limited (Suzuki et al., 2017). It includes muscarinic (miosis, hypersalivation, lacrimation, bronchorrhea, bronchoconstriction, nausea, vomiting, increased motility of the bowels, bradycardia, and hypotension), nicotinic (mydriasis, tachycardia, hypertension, fasciculations, and necrosis of skeletal muscles), and central (motor incoordination, tremors, convulsions, respiratory depression, and coma) signs of toxicity (Vale and Lotti, 2015).

The route of intoxication by nerve agents varies, but by far the most likely ones are inhalational and percutaneous. In the case of inhalational poisonings, the onset of the acute cholinergic syndrome is very fast and there is a need for equally fast administration of antidotes (Sidell and Borak, 1992; Yokoyama et al., 1996; Okumura et al., 1996). This situation occurred with tabun during the Iraq−Iran War in the 1980s (Razavi et al., 2014) and with sarin in the infamous Aum Shinrikyo sect attacks in Matsumoto, with 7 fatalities (Yoshida, 1994; Suzuki et al., 1997; Okudera et al., 1997) and in the Tokyo subway, with 12 fatalities (Suzuki et al., 1995; Nozaki et al., 1995a; Nozaki and Aikawa, 1995; Masuda et al., 1995) in 1994 and 1995, respectively. Three individual cases of assassination attempts with VX on former Aum Shinrikyo's members and anti-AUM activists happened in December 1994 and January 1995, with one fatality (Nozaki et al., 1995b; Zurer, 1998; Morimoto et al., 1999). The latest and highly profiled proven case of use of nerve agents was the assassination of Kim Jong-nam, the estranged half-brother of North Korean president Kim Jong-un in Kuala Lumpur, Malaysia, when VX was smeared across his face, causing percutaneous poisoning (BBC News, 2017).

All these incidents suggest a very real danger from the use of nerve agents both in armed conflicts and as a mean of chemical terrorism, which warrants further improvement of both prophylactic and therapeutic countermeasures (Stojiljković, 2019).

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65.2 Prophylaxis against intoxication with nerve agents

Use of standard antidotes—atropine, oximes, and diazepam—in the treatment of experimental intoxications with sarin and VX assures high protective ratios (PRs). For example, guinea pigs poisoned with sarin or VX therapy with atropine and oxime pyridinium methane sulfonate (P2S) resulted in PRs of 38 and 25, respectively (Leadbeater, 1988). A much more serious problem is represented by intoxications with tabun or soman, where, under the same conditions, very modest PRs were obtained—2.5 and 1.3, respectively (Leadbeater, 1988). It must be pointed out, however, that P2S is not an oxime that can reactivate AChE inhibited by tabun or soman (Bošković et al., 1984; Ćetković et al., 1984). Trimedoxime (TMB-4), obidoxime (LüH-6), or, more recently, oxime K203 are much better oximes against tabun poisonings (O'Leary et al., 1961; Inns and Leadbeater, 1983; Kuca et al., 2018), while oxime HI-6 achieves the best results in rodents intoxicated with soman (Bošković, 1981; Clement and Lockwood, 1982; Shih, 1993; Antonijević and Stojiljković, 2007). If we compare the PRs in rats treated with atropine and appropriate oxime intoxicated with various nerve agents, it appears that PRs obtained in animals poisoned with sarin or VX are still 5−15 times higher than those after poisoning with tabun or soman (Jokanović and Stojiljković, 2006; Stojiljković and Jokanović, 2006).

It is estimated that even well-trained military personnel can be exposed to 5 median lethal doses (LD_{50}) of soman and this is why it is expected for antidotes to be able to reach this level of protection, that is, PR 5 or higher (Dunn and Sidell, 1989). Having in mind the mentioned results for tabun and soman, the use of atropine and oxime treatment cannot meet this requirement and this is why this treatment after exposure to soman will not afford protection against more than 1.5 or 2 LD_{50} (Rickett et al., 1987). This is the main reason for the concept of adding a prophylactic antidote.

Koster (1946) was first to report on the favorable interaction between a carbamate—physostigmine—and an organophosphate (OP) AChE inhibitor—diisopropylfluorophosphate (DFP). He successfully used physostigmine as pretreatment in cats exposed to DFP and protected cats from dying after i.v. administration of 30 LD_{50} of DFP. The pretreatment regimen included a large dose of physostigmine (1 mg/kg) and a small dose of atropine (0.3 mg/kg) that was added to antagonize the muscarinic effects of physostigmine. The pretreatment interval was 3.5 h. Thereafter, Koelle (1946) demonstrated that physostigmine protected AChE from irreversible inhibition by DFP in rat brain homogenates in vitro and formulated that this finding is the reason for Koster's phenomenon.

Fleisher and Harris (1965) discovered that it was dealkylation of the soman−AChE complex that made it resistant to reactivation by oximes—the phenomenon known as aging of the AChE−soman complex. In this study, the $t_{1/2}$ of aging of the soman−AChE complex in vitro was only 2.2 min. The authors clearly showed that physostigmine 1 mg/kg i.v. injected 5 min after atropine 10 mg/kg i.v. and 15 min before soman s.c. assured a PR of 3.8 (relative to atropine alone). They also showed that after the challenge with soman 1.5 LD_{50} s.c., all the rats pretreated with physostigmine survived and were without any symptoms of poisoning, in comparison with only 50% of those animals pretreated with atropine alone. The difference in brain AChE activity 24 h after soman challenge between these two groups was also significant—58.5% versus 15.4%, respectively. What is even more important, it was shown that the aging reached a maximum 30 min after soman intoxication, when in nonpretreated rats 84.6% of all AChE activity was inhibited, of which 50% belonged to the aged portion of the enzyme, while in rats pretreated with physostigmine these percentages were much lower—41.5% and 6%, respectively. The authors concluded that the preventing by physostigmine of the interaction between soman and AChE is a very successful way to avoid not only its inhibition by soman, but also to decrease the aging of the soman−AChE complex (Fleisher and Harris, 1965).

Further investigation in the field of potential prophylactic antidotes ensued and best protection was obtained with pyridostigmine in guinea pigs (Berry and Davies, 1970; Gordon et al., 1978). Definite affirmation of pyridostigmine prophylaxis was confirmed when in experiments in guinea pigs poisoned with tabun, sarin, soman, or VX and treated with atropine, various oximes and diazepam PRs of 76, 380, 20, and 410 were obtained, respectively (Inns and Leadbeater, 1983).

The essence of this concept is reversible inhibition of a portion of AChE by a carbamate, which protects these molecules of AChE from being irreversibly inhibited by DFP or any other nerve agent (Koelle, 1946). In this case, inhibition of the remaining AChE activity by an OPC creates a nonlife-threatening situation, since over the time carbamate dissociates—decarbamylates the active center of AChE, in quantities sufficient to maintain normal transmission in cholinergic synapses (Dirnhuber and Green, 1978). The opposite sequence of administration—OPC first and carbamate second—only potentiates the cholinergic toxic effects (Koster, 1946; Takahashi et al., 1987), since carbamate inhibits the portion of AChE that remained uninhibited by the OPC (Green, 1983).

So far, more than 50 compounds have been investigated as potential prophylactic agents against poisoning with nerve agents. Some of them have been abandoned, others are still under the investigation, while some were

introduced in the corresponding equipment of modern armies. A survey of such medicines is contained in Table 65.1.

65.2.1 Use of acetylcholinesterase inhibitors in prophylaxis of poisoning with nerve agents

The most successful prophylactic schemes include carbamate AChE inhibitors physostigmine (Koster, 1946; Berry et al., 1971; Harris and Stitcher, 1984; von Bredow et al., 1991b), neostigmine (Berry et al., 1971; Heyl et al., 1980), pyridostigmine (Berry et al., 1971; Dirnhuber et al., 1979; Hauser et al., 1981; Inns and Leadbeater, 1983; Harris and Stitcher, 1984; Maxwell et al., 1988; von Bredow et al., 1991a), and some newer synthetic carbamates, like ferrocene carbamate (Gordon et al., 1978; Karlsson et al., 1984).

65.2.1.1 Physostigmine

Physostigmine, also called eserine, is a *N*-monomethyl carbamate isolated from the Calabar bean (*Physostigma venenosum* Balfour) by Jobst and Hesse (1864) and de novo synthesized by Julian and Pikl (1935). Physostigmine reversibly inhibits AChE in mammalian peripheral tissues and in the brain (Deyi et al., 1981; Harris and Stitcher, 1984). As a consequence, when administered as a triple prophylactic regimen with atropine and mecamylamine, it provides protection against 2.6 LD_{50} of soman in rats (Harris et al., 1980). Under the same conditions, this alkaloid assures a PR of 6.9 against DFP poisoning in rats (Harris and Stitcher, 1984). With the addition of atropine alone or with atropine and P2S, physostigmine pretreatment exerts similar efficacy in other species intoxicated with soman, and especially in guinea pigs, where the attained PR of the triple prophylactic regimen reaches 10.7 (Berry et al., 1971). It was confirmed that this antidotal effect is based on protection of AChE from soman-induced irreversible inhibition in vital organs (Deyi et al., 1981).

Physostigmine owes its prophylactic efficacy to the fact that it readily passes the blood–brain barrier, enters the CNS, and binds reversibly to the brain AChE, which is the main target of soman and other nerve agents (Somani and Khalique, 1986; Somani, 1989; Somani and Dube, 1989). This readiness to access the brain centers is, however, also a potential drawback of physostigmine, since it can induce some incapacitating central effects (Stojiljković et al., 1989) and for this reason it is frequently combined with small doses of lipophilic anticholinergics, such as scopolamine or procyclidine (Myhrer et al., 2013).

65.2.1.2 Neostigmine

Neostigmine is a quaternary *N,N*-dimethyl carbamate synthesized by Aeschlimann and Reinert (1931) as a physostigmine analog. Neostigmine rapidly, due to lack of central effects, replaced physostigmine in the treatment of myasthenia gravis (Remen, 1932; Walker, 1934; 1934–1935). Although its hydrophilicity and exclusively peripheral action dictated its use with central and peripheral antimuscarinic atropine and predominantly central antinicotinic mecamylamine, neostigmine had good results in prophylaxis of soman intoxications (Harris et al., 1980). From a practical point of view, neostigmine is inferior to peripherally acting carbamate pyridostigmine because of stronger stimulation of the digestive tract, which is unfavorable for a prophylactic agent.

65.2.1.3 Pyridostigmine

Pyridostigmine is an analog of neostigmine, also with *N, N*-dimethyl carbamate structure, synthesized by Urban and Schnider in 1945 (Randall et al., 1955). The frequency and severity of cholinergic adverse effects (i.e., nausea, vomiting, increased salivation, diarrhea, and abdominal cramps) of pyridostigmine are lower than in neostigmine (Duphar, 1987). Although pyridostigmine also does not penetrate the brain (Birtley et al., 1966), it is among the potential prophylactic agents and by far the most tested one, owing to its much longer prophylactic interval (4 h) than physostigmine (Gordon et al., 1978). If administered 30 min before the nerve agent and if supplemented by postexposure therapy consisting of atropine and oxime P2S, pyridostigmine produces PRs of 22, 21.5, 8, and 26.3 in guinea pigs poisoned with tabun, sarin, soman, or VX, respectively (Gordon et al., 1978). Pyridostigmine pretreatment 0.2 mg/kg and postexposure treatment with atropine protect monkeys against 28 LD_{50} of soman (Dirnhuber et al., 1979). In guinea pigs poisoned with nerve agents, the best protection was provided by pretreatment with pyridostigmine and treatment with atropine, oxime, and diazepam. Extremely high PRs were thus obtained—for tabun 76, for sarin 370, for soman 20, and for VX 410 (Inns and Leadbeater, 1983).

The Czech Army developed a special prophylactic antidote PANPAL designed to overcome the shortcomings of pyridostigmine (Kassa, 2006). It contains two tablets—PANPAL A with benactyzine hydrochloride 8 mg and trihexyphenidyle 6 mg, and PANPAL B containing pyridostigmine bromide 35 mg (Bajgar et al., 2019). The content of the first tablet, consisting of very liposoluble anticholinergics that gain rapid access to the brain (Stojiljković et al., 1989) assures early occupation of cholinoceptors in the CNS to counteract the effects of the accumulating acetylcholine, while at the same time, pyridostigmine from the second tablet protects from inhibition

TABLE 65.1 Prophylactic agents against organophosphorus compounds (OPC).

Mechanism of action	Class of agents	Prophylactic agent	References
Reversible AChE inhibition	Carbamates	Physostigmine, pyridostigmine	Dirnhuber et al. (1979), Heyl et al. (1980), Deyi et al. (1981), Inns and Leadbeater (1983), Harris et al. (1984), Leadbeater et al. (1985), Lennox et al. (1985), Shiloff and Clement (1986), Solana et al. (1990a,b), von Bredow et al. (1991a,b)
		Mobam, decarbofuran, neostigmine	Gordon et al. (1978), Harris et al. (1980), Heyl et al. (1980)
		Ferrocene carbamate	Karlsson et al. (1984)
	Aminophenols	Eseroline	Galli et al. (1985)
	Aminoacrydines	Tetrahydroamminoacridine	Galli and Mori (1991)
	Analgesics	Meptazinol	Galli and Mazri (1988)
	Plant alkaloids	Huperzine A, galantamine	Albuquerque et al. (2006), Haigh et al. (2008), Mamczarz et al. (2011), Wang et al. (2011), Hamilton et al. (2017)
	Noncompetitive AChE inhibitors	Donepezil	Janowsky et al. (2005)
Irreversible, oxime-sensitive AChE inhibition	OPC	Tetraethylpyrophosphate	Berry et al. (1971)
		Paraoxon	
		Ethyl-4-nitrophenylmethylphosphonate	
Blockade of cholinoceptors	Antimuscarinics	Atropine	DeCandole and McPhail (1957)
		Aprophen	Leadbeater et al. (1985)
		Azaprophen	Gennings et al. (1990)
		Scopolamine	Anderson et al. (1991), Lim et al. (1991), von Bredow et al. (1991b)
		Trihexyphenydyl	Berry et al. (1971), Kassa and Vachek (2002)
		Benactyzine	
	Antinicotinics	Pentamethonium	Heyl et al. (1980), Kassa and Vachek (2002)
		Mecamylamine	Berry et al. (1971)
		D-Tubocurarine	Heyl et al. (1980), Harris et al. (1980, 1984), Patterson et al. (1988)
Decreased synthesis/release of acetylcholine	Quinuclidines	*N*-Allyl-3-quinuclidinol	Sterling et al. (1988)
		Clonidine	Aronstam et al. (1986)
AChE reactivators	Oximes	Pralidoxime salts	Crook et al. (1962), Quinby (1968), Wolthuis et al. (1981)
		Pro-2-PAM	Clement (1979)
		Obidoxime	Schoene et al. (1985)
		HI-6	Schoene et al. (1985), Bokonjić et al. (1987), Shih et al. (1991b), Bajgar (2004), Bajgar et al. (2009)
		K033, K048	Lucić Vrdoljak et al. (2006)
		K27	Lorke and Petroianu (2019)

(*Continued*)

TABLE 65.1 (Continued)

Mechanism of action	Class of agents	Prophylactic agent	References
Treatment of convulsions	Benzodiazepines	Diazepam	Lundy et al. (1978), Doebler et al. (1985)
		Clonazepam	Lipp (1974)
Stoichiometric scavengers	Esterases	Acetylcholinesterase	Wolfe et al. (1987), Maxwell et al. (1992)
	Immunoglobulins	Butyrylcholinesterase	Raveh et al. (1993), Allon et al. (1998), Ševalova et al. (2004), Saxena et al. (2011, 2015), Rosenberg et al. (2014), Reed et al. (2017), Myers (2019)
		Monoclonal antibodies	Lenz et al. (1984), Rong and Zhang (1990)
Catalytic scavengers	OPC bioscavengers	Paraoxonase-1	Lenz et al. (2007), diTargiani et al. (2010), Valiyaveettil et al. (2011, 2012), Kuca et al. (2013), Worek et al. (2014)
		Prolidase	Endo et al. (1988), diTargiani et al. (2010), Kuca et al. (2013), Iyer et al. (2015)
		Senescence marker protein-30	Kondo et al. (2004), diTargiani et al. (2010), Kuca et al. (2013), Iyer et al. (2015)
		Phosphotriesterase	Tuovinen et al. (1996), Petrikovics et al. (2000), Kuca et al. (2013), Iyer et al. (2015), Poirier et al. (2018)
Protection of AChE/ blockade of glutamate receptors	NMDA antagonists	Ketamine	Clinton et al. (1988)
		Memantine	Braitman and Sparenborg (1989), Gupta and Dettbarn (1992), McLean et al. (1992), Löscher and Hönack (1994)
		Dizocilpine (MK-801)	Shih et al. (1991a), Sparenborg et al. (1992)
	AMPA antagonists	NBQX	Löscher and Hönack (1994)
	NMDA/AMPA antagonists	Procyclidine	Choi et al. (2004), Cho et al. (2012)

of AChE in the peripheral tissues (Kassa, 2006). Experiments in rats and mice poisoned with tabun and treated with standard antidotes atropine, obidoxime, and diazepam, became more resistant to the tabun-induced lethality when they were pretreated with pyridostigmine, benactyzine, and trihexyphenidyle than when the pretreatment contained pyridostigmine only (Kassa and Vachek, 2002).

65.2.2 Prophylactic use of oximes

Prophylactic use of oximes has not attracted a lot of interest, although some experiments with osmotic minipumps that maintained constant plasma concentrations of pralidoxime or asoxime strongly potentiated the PRs (up to 72) of postexposure treatment with atropine and diazepam against an OP insecticide quinalphos in dogs (Bokonjić et al., 1987). An attempt to develop asoxime tablets with regular and retarded absorption was unsuccessful, because of very low oral bioavailability of asoxime—5.42% and 8.07% for classic and retard tablets, respectively (Maksimović et al., 1987). Further applied research in this area enabled the Czech Army to produce TRANSANT, a transdermal patch with asoxime (Bajgar et al., 2009; 2019).

65.2.3 Use of *N*-methyl-D-aspartate-receptor-blocking drugs in prophylaxis against organophosphorus compounds

Memantine hydrochloride (1-amino-3,5-dimethylaminoadamantane) is an adamantine derivative used in various conditions affecting the central nervous system (CNS) (Wesemann et al., 1983). It acts as a noncompetitive antagonist of *N*-methyl-D-aspartate (NMDA) receptors for excitotoxic transmitter glutamate (Bormann, 1989; Kornhuber et al., 1989, 1991), which explains its neuroprotective effect (Seif el Nasr et al., 1990; Erdö and Shäfer, 1991). Gupta and coworkers in the late 1980s started a series of experiments, where memantine 18 mg/kg s.c. was successfully

used along with atropine 16 mg/kg s.c. as pretreatment against convulsions, muscle necrosis, and death induced by intoxications with tabun, sarin, soman, or VX (Gupta and Dettbarn, 1992; McLean et al., 1992). It was shown that memantine pretreatment decreased four times the ED_{50} of HI-6 for an antilethal effect in mice poisoned with soman, by counteracting the soman-induced convulsive activity (Antonijević et al., 2011).

Memantine pretreatment shows dose- and time-dependency. In a separate set of experiments, it was confirmed that only doses of 18 mg/kg and above (36 and 72 mg/kg) significantly potentiate the atropine/HI-6 PR against soman in rats. When an appropriate dose is selected, the pretreatment interval of memantine can be even nonexistent (0 min, meaning that it can be successfully used as a posttreatment) or as long as 8 h before soman exposure. The highest PR of 10.11 was assured with memantine 36 mg/kg injected s.c. 60 min before soman (Stojiljković et al., 2019).

Procyclidine has a wider portfolio of activities; it blocks muscarinic, nicotinic, and NMDA receptors. Its use as a prophylactic antidote along with physostigmine prevented the occurrence of convulsions induced by up to 2 LD_{50} of soman (Myhrer et al., 2004). It is combined with physostigmine and these two prophylactic drugs, administered prophylactically for 3 days via osmotic minipumps protected rats from the lethal, convulsant, and CNS-damaging effects of soman (Choi et al., 2004). The same combination was used in dogs poisoned with soman, but delivered from transdermal patches allowing for continuous liberation of active substances into the bloodstream. In this experiment, postexposure therapy with atropine and asoxime assured a PR of 2.7, prophylactic patch 4.7, and their combination 9, showing a clear case of potentiation among these prophylactic and postexposure antidotes (Kim et al., 2005). These results imply that a physostigmine/procyclidine patch might be a good alternative for pyridostigmine prophylaxis and subsequent use of diazepam (Cho et al., 2012).

Procyclidine prophylaxis is obviously dose-dependent (Fig. 65.1). An increase in its dose allows for not only survival after increasing doses of soman, but also prevents the occurrence of soman-induced convulsions. In this particular experiment, procyclidine, administered 30 min before soman along with a fixed dose of physostigmine of 0.1 mg/kg i.p. was given in doses of 1, 3 and 6 mg/kg i.p., while the doses of soman tested were 1.3, 1.6, and 2 LD_{50} (Myhrer et al., 2004).

As shown in Fig. 65.1, if a sufficiently high dose of procyclidine was injected prophylactically (6 mg/kg),

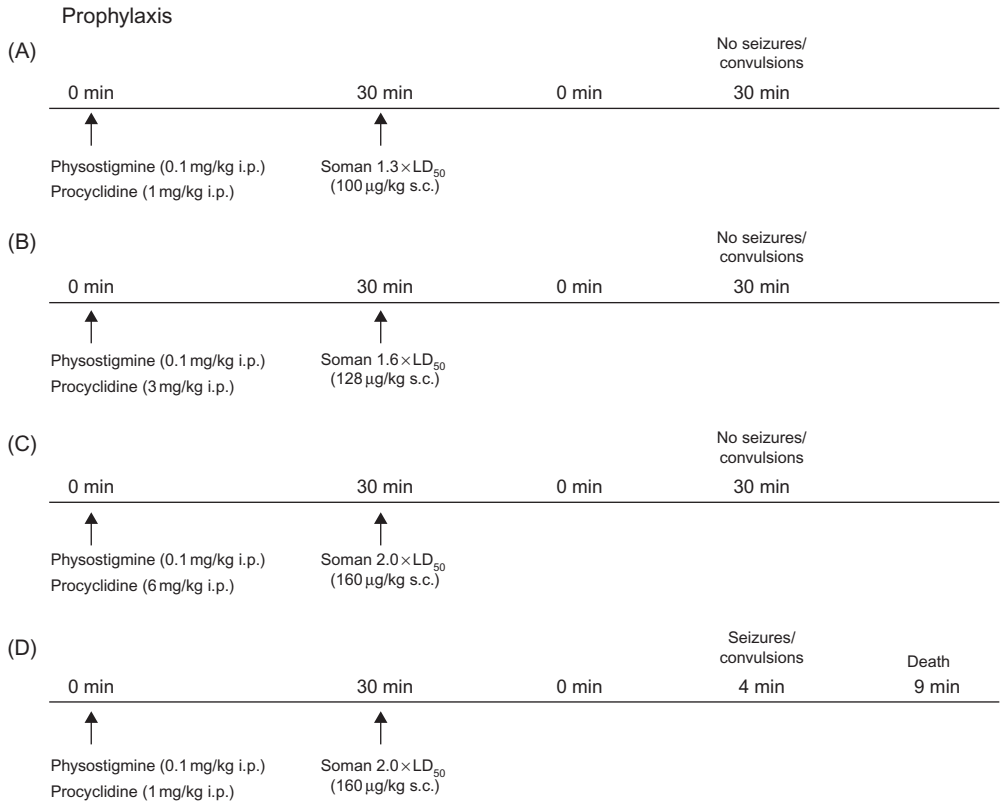

FIGURE 65.1 Experimental design for demonstration of anticonvulsant effects of a fixed dose of physostigmine combined with increasing doses of procyclidine relative to increasing doses of soman (A–C). Insufficient dose of procyclidine compared with soman results in early death (D).

even the highest dose of soman (2 LD_{50}) did not cause convulsions and death, while if the dose of procyclidine was low (1 mg/kg), the same dose of soman would in 4 min result in convulsions and, after 9 min, death. The dose-dependency of the prophylactic dose of procyclidine follows the concentration-dependent binding of procyclidine at the phencyclidine site at the *N*-methyl-D-aspartic acid (NMDA) receptor that leads to their blockade (Myhrer et al., 2004).

65.2.4 Adverse effects of prophylatic regimens

The mentioned requirement that a successful candidate for a prophylactic drug against OPC and especially nerve agents—to be able to protect against at least 5 LD_{50} of the OPC in question— is not the only one. Another aspect is safety, which means that an ideal prophylactic drug should not impair the physical and psychological functions of the human body. The main controversy is in the fact that the agents that act not only peripherally (i.e., outside the CNS), but also gain access to the brain, are the most efficient prophylactic drugs against OPCs, since the CNS is the main point of their attack. At the same time, however, such prophylactic antidotes tend to adversely affect the normal brain functions, at least at a dose that affords effective prophylaxis. For example, even the lowest dose of memantine tested—1 mg/kg s.c.—significantly increases spontaneous motoric activity in rats (Stojiljković et al., 2019).

When it comes to centrally acting carbamates, such as physostigmine, they themselves have AChE-inhibiting properties, which can be partly compensated by combining them with low doses of very lipid-soluble anticholinergics (e.g., procyclidine, scopolamine, benactyzine, trihexyphenidyl, aprophen, etc.). Even these small doses of anticholinergics per se have some adverse effects of their own, which complicates the overall tolerability of such a prophylactic regimen. For example, Sket (1993) showed that in female rats pretreated with physostigmine the same supralethal dose of soman induces the first signs of poisoning earlier than in nonpretreated rats. As a general rule, practically all pretreatment drugs tested so far, that is, physostigmine, galantamine, benactyzine, trihexyphenidyl, procyclidine, and even only peripherally acting carbamate pyridostigmine, induce per se some level of incapacitation of the laboratory animals at doses effective against nerve agents, which represents a serious problem in the development of prophylactic antidotes (Myhrer and Aas, 2016). A possible strategy would be to give prophylactically only moderate doses of drugs and compensate them later, during the treatment of poisoned individuals (Myhrer et al., 2004).

65.2.5 Bioscavengers against nerve agents

Nerve agent bioscavengers can be defined as biological molecules—proteins and enzymes—that may prevent intoxication by binding and inactivating nerve agents before they can reach their target organs. They can be divided into two classes: stoichiometric and catalytic bioscavengers. Stoichiometric bioscavengers "sacrifice themselves" by binding irreversibly to molecules of nerve agents, while catalytic bioscavengers destroy the molecules, remaining intact during the process (Goldsmith and Ashani, 2018). Currently, only Protexia is available for human use (Bajgar et al., 2019). It is a pegylated form of a recombinant human butyrylcholinesterase (Mumford and Troyer, 2011) that becomes 50% inhibited in vitro when added to a nerve agent in a stoichiometric ratio of 1:1 (Cerasoli et al., 2005). It was successfully tried in vivo in guinea pigs percutaneously poisoned with VX 2.5 LD_{50}, when Protexia, injected i.m. 2 h after exposure to VX, prevented death and alleviated signs of cholinergic poisoning in all exposed animals (Mumford and Troyer, 2011). Human butyrylcholinesterase seems to be behaviorally safe in monkeys per se, and after challenging them with an otherwise lethal dose of soman (Myers, 2019).

Even better results are expected from catalytic scavengers. Human paraoxonase-1 (PON-1) is certainly one of them, albeit its affinity for OPCs is only moderate and the turnover rate is slow. Therefore, prophylactic use of native human PON-1 cannot result in dramatic protective effects against nerve agents (Lenz et al., 2007). For this reason, further efforts were invested in the development of mutants of human PON-1 (Yeung et al., 2004; Aggarwal et al., 2016). Although the first efficacy results are favorable (Worek et al., 2014), their immunogenicity and plasma half-lives in humans have yet to be determined (Lenz et al., 2007).

65.3 Treatment of intoxication with nerve agents

Current principles of postexposure treatment of poisonings with nerve agents include a triple regimen, that is, use of causal antidotes—AChE reactivators and of two classes of symptomatic antidotes—anticholinergics and anticonvulsants.

65.3.1 Anticholinergics

Although this class of medicines acts as symptomatic antidotes by blocking cholinergic receptors in the periphery and in the CNS, they represent the basis of any effective treatment of nerve agent poisonings, in experimental animals and in humans alike. Among them, a subclass of antimuscarinics is significantly more important than the

other, antinicotinics. The reason for that is because primarily overstimulation of muscarinic receptors is responsible for the suppression of the activity of vital centers in the brain (Škrbić et al., 2017).

Among them, atropine is by far the best known and most widely used antimuscarinic. Although it passes through the blood–brain barrier into the brain, its relative lipophilicity is low in comparison with the other antimuscarinics, such as scopolamine, benactyzine, and aprophen (Stojiljković et al., 1989). Atropine assures high and dose-dependent PR against carbamate AChE inhibitors and especially those that act on this enzyme in the brain, like physostigmine (Stojiljković et al., 2018). At the same time, antinicotinics hexamethonium and d-tubocurarine are much more efficient against strictly peripherally acting carbamates, like pyridostigmine (Stojiljković et al., 2018). Nevertheless, there is only modest protection against OPCs and nerve agents (if any) by atropine monotherapy (Inns and Leadbeater, 1983; Leadbeater et al., 1985). Addition of antinicotinics increases PRs primarily against carbamates, but also to a limited extent also against OPCs (Parkes and Sacra, 1954). Recent results confirm that a bispyridinium antinicotinic compound MB327 protects guinea pigs from soman poisoning when administered along with atropine and avizafone (Price et al., 2018). Experiments with the same compound in mice intoxicated with tabun, sarin, or soman showed that addition of MB327 increases the LD_{50} of all three nerve agents treated with atropine or atropine and asoxime. This increase, however, reached statistical significance only in the case of sarin (Kassa et al., 2016).

65.3.2 Acetylcholinesterase reactivators

This class of drugs act as causal antidotes, since they reactivate AChE irreversibly inhibited by OPCs and in this way remove the cause of the poisoning. They all contain the oxime functional group in their molecules and this is why they are also called oximes. The older monopyridinium oximes, such as pyridine-2-aldoxime dichloride (2-PAM) and its methanesulfonate salt (P2S), are effective reactivators of sarin- and VX-inhibited AChE and hence their combinations with atropine assure reasonably high PRs against these two nerve agents. At the same time, they are totally ineffective against tabun and soman. The next generation were trimedoxime (TMB-4) and obidoxime (LüH-6) that are effective reactivators of tabun- but not soman-inhibited AChE, with good antidotal efficacy against tabun, sarin, and VX.

Asoxime (HI-6) was one of the products of Ilse Hagedorn's laboratory, known as H-oximes, and it was the first oxime known to reactivate AChE previously inhibited by soman, the nerve agent that represented the most difficult target for treatment. Although also a bispyridinium oxime with low lipophilicity, it gains some access into the CNS in concentrations high enough to reactivate AChE and in this way act as a true causal antidote (Ligtenstein and Kossen, 1983; Ligtenstein et al., 1988). Use of the atropine/asoxime combination, with or without diazepam, therefore became the gold standard of antidotal treatment against soman (Pantelić and Maksimović, 1982; Jokanović and Stojiljković, 2006; Stojiljković and Jokanović, 2006). In order to cover all four major nerve agents, a combination of asoxime and obidoxime was tried, with good results against inhibition of AChE induced by tabun, sarin, soman, VX, and cyclosarin (Worek et al., 2007).

All H-oximes obviously have non-AChE-mediated effects known as "direct pharmacological effects" (Van Helden et al., 1996; Jokanović and Prostran, 2009; Jokanović, 2012). The existence of these not well elucidated antidotal mechanisms is obvious, because without them it would be impossible to explain findings like that of the Canadian authors who registered PR as high as 5 in tabun-poisoned guinea pigs treated with atropine and asoxime 125 mg/kg i.p., that is, with an oxime that cannot reactivate tabun-inhibited AChE (Hamilton and Lundy, 1989). Asoxime therefore became a component of dual and triple autoinjectors, the other two components being atropine and avizafone, a hydrosoluble version of diazepam. Due to asoxime's instability in water, it has to be stored as a powder and dissolved in atropine solution practically upon the autoinjector activation (Bajgar, 2010).

The H-oxime with broadest reactivation specter is HLö-7 (Löffler, 1986), an oxime that can reactivate AChE inhibited by all four major nerve agents, tabun, sarin, soman, and VX, but also the one inhibited by cyclosarin (DeJong et al., 1989; Worek et al., 1994a,b, 1995; Lundy et al., 1992). On the protection level, it proved to be more effective than asoxime against tabun and VX, while asoxime was more effective than HLö-7 in animals poisoned with sarin, soman, and cyclosarin (Lundy et al., 1992; Eyer et al., 1992). Despite its broad spectrum, it never became a candidate for autoinjectors due to several factors that include greater per se toxicity than asoxime, difficult synthesis, and instability in water solutions (Dawson, 1994).

K-oximes, named after two Czech researchers Kamil Kuča and Kamil Musilek, represent a potentially important step forward (Musilek et al., 2005). These oximes, and especially K-203, can reactivate even tabun-inhibited AChE. Their commercial exploitation however seems currently to be a remote possibility (Musilek et al., 2007).

It is important to underline that the oximes used currently by military personnel—2-PAM, P2S, obidoxime, and asoxime—although they also come in monocomponent autoinjectors, are generally used only as an addition to atropine, since their PRs when used alone are modest.

65.3.3 Anticonvulsants

The toxic accumulation of acetylcholine that follows AChE inhibition in brain triggers convulsions that induce the occurrence of neuronal death and structural lesions of the CNS on one hand and compromisation of the function of the respiratory center leading to central respiratory paralysis. Numerous studies have shown that early administration of atropine, but also of some other antimuscarinics or/and antinicotinics, can prevent the occurrence of seizures. However, once the seizures become fully developed, they lose sensitivity to anticholinergics and can be stopped only after administration of anticonvulsants (Myhrer et al., 2004).

Two classes of anticonvulsants were used against nerve agents—benzodiazepines and glutamate receptor antagonists.

65.3.3.1 Benzodiazepines

Although the addition of oximes to atropine potentiates its PR, the inclusion of benzodiazepines into a triple regimen usually results in further potentiation of PR in animals poisoned with nerve agents. Their mechanism of action includes stimulation of the frequency of opening of $GABA_A$ receptors that results in the influx of chloride anions and in hyperpolarization of cell membranes in the CNS. As a result, neurons become less susceptible to the generation of seizures (Sellström, 1992; Jokanović, 2015).

Among the benzodiazepines, diazepam is most frequently used. Its drawback is its insolubility in water and hence inability to be packed in an autoinjector, but also a relatively slow onset of action. Experiments in rodents have shown that any delay in administration of diazepam after exposure to soman significantly diminishes its anticonvulsive efficacy (McDonough et al., 2010). Consequently, even a diazepam dose of 20 mg/kg could not treat convulsions induced by 1.6 LD_{50} of soman when injected more than 40 min after soman (Shih et al., 1999).

Avizafone (lysyl peptido-amino-benzophenone diazepam or pro-diazepam) is a hydrosoluble complex that liberates diazepam and can be used in autoinjectors (Clair et al., 2000). It was shown in a clinical trial that avizafone, after injection, assures significantly higher concentrations of diazepam in plasma than diazepam itself and that this maximum is reached earlier (Abbara et al., 2009). On the efficacy level, avizafone performed better than an equimolar dose of diazepam, when administered with atropine to guinea pigs poisoned with sarin (Taysse et al., 2003) or soman (Taysse et al., 2006). Armies of the United States, Canada, and Norway have autoinjectors with diazepam, while the United Kingdom and the Netherlands use autoinjectors with avizafone (Aas, 2003).

There is some experience with midazolam, a very potent hydrosoluble benzodiazepine, that has faster onset of action and potentiates better the PRs of atropine/asoxime combinations in rodents intoxicated with nerve agents (Bokonjić and Rosić, 1991; McDonough et al., 1999, 2009). Other benzodiazepines, like clonazepam, were rarely tried against nerve agents (Lipp and Dola, 1980; Shih et al., 1991b).

65.3.3.2 Glutamate receptor antagonists

Most of the drugs belonging to this class act as NMDA receptor antagonists, although NBQX actually blocks another two classes of ionotropic glutamate receptors—AMPA (quisqualate) and kainite receptors (Löscher and Hönack, 1994; Filliat et al., 1998). Ketamine, dizocilpine (MK-801), phencyclidine (PCP), gacyclidine (GK-11), tenocyclidine, and memantine belong to this therapeutic group (Shih et al., 1991b; Dorandeu et al., 2005). Their mechanism of action is simple—they block noncompetitively an open channel of NMDA receptors and thus prevent them from overstimulation by the excitotoxic amino acid glutamate. The resulting effect is anticonvulsant and neuroprotective. Representatives of this class were used successfully to prevent or stop soman-induced convulsions.

Tenocyclidine, a phencyclidine derivative, and its adamantane derivatives, augmented the protection afforded by atropine and asoxime against soman in mice, assuring PRs within the range of 5.40−7.12 (Skare et al., 2002). In a separate experiment, tenocyclidine was administered to guinea pigs pretreated with pyridostigmine, poisoned with 2 LD_{50} of soman and treated with atropine. In this experiment, tenocyclidine was able to block the already existing status epilepticus, even if administered 60 min after the onset of convulsions (Carpentier et al., 1994).

Gacyclidine was injected in pyridostigmine-pretreated monkeys 45 min after they were poisoned with 8 LD_{50} of soman and treated with atropine/2-PAM/diazepam. Gacyclidine increased survival, and normalized the EEG activity. In addition, 3 weeks after poisonings, serious neuropathological sequelae were found in animals that did not receive gacyclidine, but not in those ones that did. This was strong proof of the antidotal, anticonvulsant, and neuroprotective effect of gacyclidine against soman intoxication (Lallement et al., 1997, 1998).

Of possible importance for further research is the finding that caramiphen, a compound with combined anticholinergic and anti-NMDA properties, stops the soman-induced seizures, decreases neuronal loss, and neuronal degeneration even when administered 30 or 60 min after soman (Figueiredo et al., 2011). Both caramiphen and procyclidine can be combined with the well-known antiepileptic drug levetiracetam to stop soman-induced seizures in rats, when applied either before or after soman (Myhrer et al., 2011).

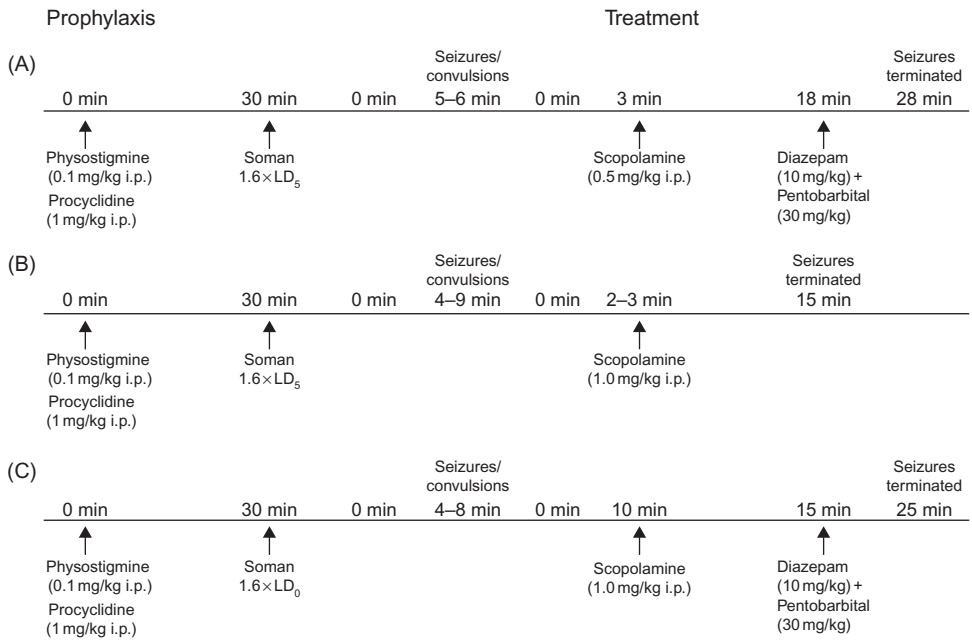

FIGURE 65.2 Experimental design for demonstration of how insufficient prophylactic treatment can be compensated for by adjunct postexposure treatment. A low dose of scopolamine requires additional treatment (A). A high dose of scopolamine is sufficient to terminate seizures (B). A high dose of scopolamine given outside the cholinergic window requires additional treatment (C).

It was later shown in nonpretreated primates that even when the postsoman exposure therapy atropine/2-PAM/diazepam is delayed for 30 or 45 min, gacyclidine was able to stop the soman-induced seizures and prevent neuropathological changes from developing (Lallement et al., 1999a,b). Toxicity of gacyclidine is lower than in other NMDA receptor antagonists and is believed that it results from its binding to some non-NMDA receptor sites (Hirbec et al., 2001).

The latest experimental results indicate that soman-induced convulsions induce a "maladaptive trafficking" between $GABA_A$- and glutamate receptors in the brain, leading to a decrease of the number of $GABA_A$-receptors and an increase of the population of NMDA- and AMPA-receptors. The addition of NMDA-receptor-blocking agent ketamine to midazolam increased its anticonvulsant potential against soman. However, if postponed for 40 min after soman injection, only ketamine seems effective. According to this receptor trafficking hypothesis, diminishing efficacy of benzodiazepines that follows the duration of the untreated seizures is the result of the shift in sizes between the $GABA_A$- and NMDA-/AMPA-receptor populations (Niquet et al., 2019).

65.3.3.3 Time- and dose-dependency of administration of drugs for prevention and treatment of nerve agent-induced convulsions

The occurrence of convulsions following acute intoxication with soman or other nerve agents depends on the presence and dosage of prophylactic antidotes (e.g., memantine, physostigmine, and procyclidine) and of the nature and timing of the administration of postexposure antidotes. As mentioned earlier, the use of qualitatively and quantitatively adequate prophylactic antidotes in rats exposed to soman up to 2 LD_{50} eliminates the need for postexposure therapy (Fig. 65.1; Myhrer et al., 2004). In the case of administration of inadequately low doses of prophylactic procyclidine (1 mg/kg), convulsions leading to quick death would ensue and postexposure treatment becomes absolutely necessary (Fig. 65.2).

In the experiment presented here, 1.6 LD_{50} of soman was used and scopolamine, a very liposoluble antimuscarinic drug, had to be used, but its effect depended on its dose and time of administration. When its dose was 1 mg/kg i.p. and when it was injected 4−9 min after the occurrence of convulsions, they ceased after another 15 min. When the dose of scopolamine was lower (0.5 mg/kg) and the timing remained the same as in the previous experiment, the convulsion would be stopped only after administration of diazepam (10 mg/kg) and pentobarbital (30 mg/kg). The same combination had to be used to stop the soman-induced convulsions even after 1 mg/kg of scopolamine, but administered 10, instead of 3 min after the onset of convulsion (Fig. 65.2; Myhrer et al., 2004).

As mentioned earlier, even the use of benzodiazepines has a narrow window of effectiveness following intoxication with nerve agents, since with the passage of time these convulsions became more dependent on the glutamatergic receptor activity and relatively independent on the activity of cholinergic receptors and even the $GABA_A$/benzodiazepine-receptor complex. As a result of

this process, glutamatergic receptor antagonists remain the only solution for the delayed treatment of such convulsions (Lallement et al., 1999a,b; Niquet et al., 2019).

65.3.3.4 Autoinjectors

As mentioned earlier, modern armies try to protect their personnel against combat use of nerve agents. For this purpose, they supply their soldiers with autoinjectors, devices usually with one or two chambers that can, once activated, stick a needle into the muscle and deliver a solution containing antidotes. In this manner, precious time is saved in administering quickly needed medication. Some of these autoinjectors are monocomponent, containing only one antidote, usually atropine, an oxime, or an anticonvulsant (diazepam or avizafone). The choice of an oxime varies, depending on the specific country and its doctrinal approach to the treatment of nerve agent intoxications. For example, pralidoxime is used in the United States (2-PAM) and the United Kingdom (P2S), obidoxime in Germany, the Netherlands, Norway, and Finland, and asoxime in Canada and Sweden (Aas, 2003).

In some cases, an autoinjector contains atropine and an oxime as a combination. In the case of atopine/asoxime autoinjectors, these two components need to be stored separately due to rapid degradation of asoxime in water solutions. This problem is solved by storing the atropine solution in one chamber and the lyophilized powder of asoxime in the other (dual-chamber autoinjectors). The fact that solubility of asoxime in the form of dichloride (HI-6 Cl) is not optimal is overcome by replacing this salt with dimethanesulfonate (HI-6 DMS) that has an even better pharmacokinetic profile and certainly better water-solubility. A three-chambered autoinjector contains atropine, oxime, and diazepam (in the form of avizafone), and would be ideal for the treatment of nerve agent intoxications (Bajgar, 2010).

65.4 Concluding remarks and further directions

Further research aimed at continuous improvement of the efficacy and tolerability of prophylactic and postexposure antidotes against poisonings with nerve agents is an absolute necessity. Currently, pyridostigmine tablets represent the official prophylactic antidote in the majority of NATO countries, although the result of their use assures only partial protection, since this carbamate does not pass the blood–brain barrier and does not protect the AChE of the CNS from inhibition by nerve agents. Prophylactic regimens containing a combination of the centrally acting carbamate physostigmine and an anticholinergic could be a better solution, especially in the form of transdermal patches. As an alternative, prophylactic regimens that include the use of stoichiometric or catalytic scavengers deserve further attention.

The antimuscarinic drug atropine, monopyridinium oxime pralidoxime salts (2-PAM, P2S), bispyridinium H-oximes (trimedoxime, obidoxime, asoxime, HLö-7), K-oximes (K-207), and anticonvulsants (benzodiazepines, glutamate receptor antagonists) remain the main pillars of postexposure treatments of intoxications with nerve agents. To protect military personnel, autoinjectors are being developed, with an ideal of having atropine, universally active oxime (or a combination of obidoxime and asoxime) and diazepam, avizafone or midazolam as anticonvulsants, all in one autoinjector. Further improvement of existing autoinjectors is expected in the future.

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Chapter 66

Physiologically based pharmacokinetic/pharmacodynamic modeling of countermeasures to nerve agents

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66.1 Introduction

Physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling has proven useful in many areas of toxicology and therapeutics. This quantitative, mechanism-based approach has allowed limited experimental in vivo and in vitro data to be quantitatively integrated with physiological data to facilitate predictions of the behavior of organisms under different exposure conditions. Specifically, it has been used for:

- Extrapolation across species (particularly experimental animals to humans)
- Extrapolation across dosing scenarios (such as high to low doses)
- Extrapolation across routes of exposure

Models have been developed for a number of chemical warfare (CW) agents (Langenberg et al., 1997; Worek et al., 2005; Aurbek et al., 2006; Sweeney et al., 2006; Van der Merwe et al., 2006; see also Chapter 58: Neuropathy target esterase as a biomarker and biosensor of delayed neuropathic agents) and surrogates, including diisopropyl fluorophosphate (DFP) (Gearhart et al., 1990, 1994), diazinon (Poet et al., 2004), and chlorpyrifos (Timchalk et al., 2002a,b). In the case of agent countermeasures, PBPK/PD modeling also allows the prediction of therapeutic efficacy under varying exposure scenarios. Specifically, it may allow therapeutic regimens to be optimized to particular exposure scenarios (routes, exposure times, concentrations). To do so, PBPK/PD models of individual agents and countermeasures must be combined to take into account their interactions, whether they are pharmacokinetic or pharmacodynamic. Such modeling of agent or (multiple) counteragent combinations is an example of mixtures modeling (Krishnan et al., 1994).

There is little in literature approaching a complete model of the kinetic and dynamic interaction of nerve agent (NA) and its current (multiagent) or potential countermeasures. There have been, however, a number of important steps in that direction, both on the level of model development and in the experimental generation of data useful for the development and validation of such models.

We restrict our discussion here to countermeasures of NAs and do not discuss other chemical agents such as mustards and cyanide and their antidotes. However, the modeling ideas outlined here can also apply to other agents.

66.2 Background

Organophosphate compounds (OPs), such as the NAs, sarin (GB), soman (GD), tabun (GA), and VX, exert their toxicity via inhibiting acetylcholinesterase (AChE). As a result, Acetylcholine (ACh) accumulates in the synapses and neuromuscular junctions, resulting in hyperstimulation of muscarinic and nicotinic cholinergic receptors. Clinical symptoms include salivation, lacrimation, urination, defecation, and possibly convulsions (Myhrer et al., 2007).

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At moderate to high doses, seizures rapidly progress to *status epilepticus* (SE), which can lead to irreversible brain damage or death (Filliat et al., 1999; Carpentier et al., 2001). Survivors suffer long-lasting cognitive deficits and behavioral changes (McDonough et al., 1995) largely attributable to neuronal degeneration of hippocampal structures. Under acute high-level exposures, the focus of countermeasure treatment is the prevention of seizures, respiratory failure, and bradycardia due to convulsive spasms. For low-level exposures, and for the survivors of high exposures, the concern shifts to minimizing the cognitive impact of the agent that may or may not be related to the degree of seizure activity.

McDonough and Shih (1997) hypothesized that NA-induced seizures follow three main phases. Initially, approximately 5 minutes after exposure, hypercholinergic activity triggers the seizure. A transitional phase of cholinergic and glutamatergic hyperactivity follows as a self-sustaining glutamate−NMDA receptor (NMDAR)-mediated positive feedback loop begins to develop. After approximately 40 minutes, a predominantly glutamatergic phase sustains seizures, leading to neuronal damage predominantly in the hippocampus, amygdala, piriform cortex, thalamus, and entorhinal cortex across species (Olney et al., 1983; Wade et al., 1987; Carpentier et al., 1990; Fosbraey et al., 1990; Lallement et al., 1992; Solberg and Belkin, 1997).

High levels of glutamate are directly neurotoxic because overstimulation of glutamate ionotropic receptors allows excessive influxes of Na^{2+} and Ca^{2+}, causing prolonged depolarization of postsynaptic membranes (Bittigau and Ikonomidou, 1997). A cascade of events results from the delayed Ca^{2+} overload, leading to excitotoxic cell necrosis (McDonough and Shih, 1997; Solberg and Belkin, 1997). In addition, as the time lapse before anticonvulsant treatments increases, the Ca^{2+} influx induces transcription factors that mediate significant increases in damaging pro-inflammatory peptides, such as IL-1β, IL-6, and mRNA of TNF-α (Chapman et al., 2006).

The content of this chapter is not inclusive in the sense of taking one through the complete development and validation of a complex PBPK/PD model, which would describe the kinetics of each component, together with the pharmacodynamic interactions between selected NAs and countermeasures. Rather, this chapter briefly describes some examples of progress made in quantitative modeling and exploring how specific countermeasures interfere in this NA-induced cascade of events, and how such quantitative approaches could be used to develop improved treatment regimens.

66.3 Current countermeasures

Conventionally, NA poisoning is treated by a combination of prophylactic and post-exposure therapy, which target the three post-exposure phases of neurotransmitter systems described. Prophylactic treatments are designed to circumvent aging of the NA−AChE complex and consist of carbamate anticholinesterases (e.g., pyridostigmine) to bind AChE reversibly. Current carbamate pretreatment regimes bind 30%−40% of available red blood cell (RBC) AChE, thereby protecting some of the enzyme from irreversible OP binding (McDonough and Shih, 1997). Carbamates, however, are not without side effects. Partial AChE blockage by pyridostigmine results in a transient overstimulation of the AChR, mimicking mild NA poisoning with nausea, diarrhea, shortness of breath, and dizziness (Abraham et al., 2002). Moreover, repeated prophylactic administration of carbamates is associated with detrimental and debilitating changes in nerve and muscles function (Hudson et al., 1986).

Standard post-exposure treatments include concurrent administration of anticholinergics, such as the muscarinic cholinergic blocker atropine sulfate, and AChE reactivators, such as obidoxime and pyridine-2-aldoxime methylchloride (also known as 2-PAM). Oximes cannot reactivate OP-inhibited AChE that has already "aged." Therefore, traditional oxime treatment is considered to be less effective for those agents such as soman, for which aging is rapid (Worek et al., 2005).

If seizures develop, then treatments that attempt to attenuate the hyperglutamatergic phase are required. Anticonvulsants, such as the benzodiazepine, diazepam, may be used. Diazepam is a lipophilic agonist of the γ-aminobutyric acid A ($GABA_A$) receptor, the most predominant inhibitory receptor of the CNS. The $GABA_A$ receptor is ubiquitously distributed. Therefore, its activation or inactivation affects virtually all brain regions, making dosage critical. Treatments with traditional benzodiazepines depend on administration within a very narrow time span to be effective because profound neuropathological changes are usually detected within 20 minutes of the onset of seizures (Lallement et al., 1994; Leadbeater et al., 1985; McDonough et al., 1995; Shih et al., 1999). By themselves, benzodiazepine anticonvulsants provide limited protection, and seizures recur with desensitization of $GABA_A$ receptors (Goodkin et al., 2005). In addition, diazepam has consistently failed to eliminate the neuropathological changes resulting from NA-induced seizures (McDonough and Shih, 1997). Furthermore, in many battlefield and civilian exposure scenarios for NAs, pretreatments are unrealistic and post-exposure anticonvulsants are likely to be delayed. Epidemiological studies have shown that time to seizure treatment in the United States varies broadly, with only approximately 41% of all patients receiving their first antiepileptic drug within 30 minutes (Pellock et al., 2004).

66.4 Novel countermeasures

Because of the undesirable side effects of the standard treatment regimes, and because of limitations of

traditional benzodiazepines to adequately control seizures, the need for alternative therapeutics still exists. The focus of OP intoxication treatment is also expanding from immediate survival to include the prevention of long-term cognitive deficits. Other antidotes have been proposed that demonstrate promise in reducing excessive glutamatergic activity and/or prevent destabilization of Ca^{2+} homeostasis, because excitotoxic mechanisms of seizure activity ultimately result in Ca^{2+} overload and activate various enzymes that lead to necrosis of the cell. Attempts have been made by blocking the *N*-methyl-d-aspartate (NMDA) or alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, two subtypes of CNS glutamate receptors (Braitman, 1989; Lallement et al., 1994; McDonough and Shih, 1997; Raveh et al., 2003). NMDA-type glutamate receptors are glutamate-gated cation channels whose permeability to Ca^{2+} underlies aspects of normal synaptic plasticity. Excess Ca^{2+} influx through NMDARs mediates cell death in certain neurodegenerative pathologies. Therefore, neurons must precisely control NMDAR synaptic density, which is negatively regulated by global neuronal activity.

A number of glutamate receptor antagonists have demonstrated neuroprotective and anticonvulsive effects in animals (Urbanska et al., 1998). The high-affinity NMDAR antagonist, ketamine, has shown promise when administered intramuscularly with atropine sulfate up to 30 minutes after poisoning (Dorandeu et al., 2005). Other noncompetitive NMDA antagonists, like dizocilpine (MK-801) and *N*-(1-[2-thienyl]cyclohexyl)3,4-piperidine (TCP), display the ability to terminate seizures when treatment is delayed by 40 minutes but seriously decrease respiration (Carpentier et al., 1990; Shih, 1990; Shih et al., 1991; McDonough and Shih, 1997; Shih and McDonough, 1997).

An unusual NMDAR antagonist that recently demonstrated potent neuroprotection against GD-induced seizure-related brain damage in rats is dexanabinol. Dexanabinol is a nonpsychoactive synthetic cannabinoid that acts as a highly selective, low-affinity NMDAR antagonist (Feigenbaum et al., 1989; Eshhar et al., 1993). In GD-exposed rats ($1.6 \times LD_{50}$ subcutaneous), dexanabinol administered intraperitonealy at either 5 or 40 minutes after the onset of seizures reduced median brain lesion volume 86% and 81%, respectively (as assessed by microtubule-associated protein 2 [MAP2]-negative staining) (Filbert et al., 1999). When administered 5 minutes after seizure onset and repeated every 6 hours up to 24 hours, 98% reduction was seen. However, it is not known if the same degree of protection would be afforded if measurements were taken later than 24 hours after seizure onset. Dexanabinol did not diminish seizure activity in any of the GD-treated rats, suggesting that its neuroprotective effects may be due to its properties as a potent antioxidant and free radical scavenger, as well as its ability to inhibit the production of tumor necrosis factor-alpha (TNF-α) (Shohami et al., 1997), thus also blocking the signal transduction pathway that activates nuclear factor B (NFB).

Data to date also indicate that enzyme-based bioscavengers show much promise for the next generation of pretreatments or antidotes, with potentially no observable side effects. Candidate bioscavenger proteins either bind and sequester toxic OPs (such as serum cholinesterases and carboxylesterases) or catalytically break down the OP into nontoxic products (such as human serum organophosphorus acid anhydride hydrolases [OPAH] or paraoxonase [HuPON]). These scavengers, as well as carboxylesterase (CarbE), are each capable of providing protection against $2-16 \times LD_{50}$s of GD, GB, or VX, depending on the scavenger and the test species (rat, mouse, rabbit, guinea pig, or rhesus monkey), with little to no deficits in behavioral testing (Li et al., 2005). Human clinical trials of several bioscavengers are now underway that could lead to FDA licensure.

Human serum paraoxonase (HuPON1) is a Ca^{2+}-dependent enzyme that effectively hydrolyzes many toxic AChE-inhibiting organophosphates (Blum et al., 2008). HuPON1 is present in high-density lipoproteins of human plasma and plays an important role in protecting lipoproteins and cell membranes from oxidative damage. Because it is a human enzyme (Li et al., 2005), it could potentially be used as either a prophylaxis or medical post-treatment for NA exposure, without causing adverse immune reactions (Draganov and LaDu, 2004). Human PON1 has catalytic activity against the pesticide paraoxon and the NAs sarin (GB), soman (GD), and cyclosarin (GF), but shows only minimal activity against the nerve agent VX (Blum et al., 2008). The advantage of catalytic scavengers is the very small amount of the exogenous enzyme needed to hydrolyze and detoxify large amounts of NA.

66.5 PBPK/PD modeling

For obvious ethical reasons, only animal experiments can be used to evaluate new NA antidotes. However, the extrapolation of data from animals to humans is hampered by marked species differences. Currently, quantitative analyses addressing the co-treatment of countermeasures and medicinal treatments simultaneous with or immediately preceding or after NA exposures have been very limited. The current paradigm thus relies on experimentation in various animal models to determine efficacy and then, following the classical model for pharmaceutics, scales the animal results to the human exposure scenario. In the case of NA countermeasures, short of controlled human experimental studies, this leaves a large

uncertainty factor regarding the protection of military personnel from NA threats and may severely limit or compromise operational risk management (ORM). One solution to this dilemma is to develop validated mathematical models to predict the biological impact of simultaneous and sequential low-level exposures to specific NAs, together with specific countermeasures, countermeasure combinations, and dosing regimens.

PBPK/PD models can be used to explore the interactions between agents, countermeasures, and the organism to integrate multiple animal (in vivo and in vitro) and human (typically in vitro) data sets and to develop tools for countermeasure design and dosing regimen selection to maximize countermeasure administration efficacy. The models make quantitative use of differences in physiological parameters between test animals and humans, such as organ blood flow. The result of this approach is the development of an interaction model quantifying competition for AChE binding sites between specific agents and countermeasures, as well as other relevant pharmacodynamic and pharmacokinetic modes of interaction and the development of methods to optimize countermeasure therapies for specific NA exposure scenarios in humans.

The process of applying mathematical constructs to describe experimental results often reveals patterns in the agent's pharmacokinetics or dynamics that might not otherwise be discernable. Failure of a model's simulations to predict experimental measurements sometimes prompts questioning of the data, such as the reliability of the quantitative methods, sample collection, or exposure techniques. More often, it may indicate that greater complexity in the structure of the model is required to capture the behavior of the data. This is another primary reason for developing models, that is, to create hypotheses (model structures) that are falsifiable, leading to improved models and improved predictions in an iterative process.

There are currently a number of critical toxicological data gaps related to NA exposure at both acute high levels and repeated low levels. Many of these gaps are due to the impossibility of conducting controlled human experiments involving NA exposure. Filling these data gaps for exposure to NAs is essential for predicting performance degradation of personnel, enhancing risk assessment modeling tools, and defining detection thresholds that are physiologically relevant. The purposes of filling such data gaps are to develop a valid method for predicting dose–response effects for exposures to low NA agent concentrations/doses over long durations, and to identify threshold exposure conditions at which toxicologically significant effects occur. PBPK/PD modeling provides a means to fill these gaps. Because of the physiologically based nature of these models, simulations of experimental data can be performed by one exposure route in a particular species to develop and validate the PBPK model, and then that model can be used to simulate and predict the kinetics and pharmacodynamics in another species, such as the human exposed via another exposure route. The power of PBPK/PD models thus lies in aiding the ability of scientists and decision-makers to make reliable quantitative predictions concerning the potential health effects of real-life human exposures. In addition, this modeling approach can be useful for the development of meaningful therapeutic animal models. After all, therapeutics that have been successfully tested in animals can ultimately be tested in human clinical trials. However, their therapeutic efficacy against NAs can only be tested in animals.

66.6 Development of PBPK/PD models

Critical components to PBPK models include species-specific physiological parameters and chemical-specific parameters. Species-specific physiological parameters include the organ weights and blood flow rates for the defined compartments in the PBPK model. These values are most often available in the published literature (Brown et al., 1997; Peeters et al., 1980) and, when lacking, can be derived by appropriate scaling factors from similar species. Chemical-specific parameters may include tissue to blood partition coefficients and metabolic rate constants, which may be measured experimentally or predicted, as described in the next section on quantitative structure–activity relationship (QSAR) methods. The basic structure of a PBPK model used to describe pharmacokinetics and pharmacodynamics of a compound is similar to the model presented in Fig. 66.1, which predicts the absorption, distribution, metabolism, and excretion (ADME) of the oxime, TMB-4 (Sterner et al., 2013).

Although PBPK models can be used to predict ADME, they can be expanded to quantitatively describe the compounds pharmacological mechanism(s). Fig. 66.2 shows a schematic representation of the pharmacodynamic processes involved in the interactions between NAs such as sarin with the organism (via AChE inhibition), together with interactions with pretreatments (pyridostigmine bromide [PB]) and countermeasures (oxime, atropine, and diazepam). When this PD submodel is linked to the PBPK model via the simulated concentration of a particular NA or therapeutic in a selected target tissue (i.e., plasma, RBCs, diaphragm, and brain), it provides a framework for describing these biochemical processes quantitatively. Linking it with the PBPK model also puts the biochemical processes into a physiological context. This allows extrapolation of results to multiple species, to description of relevant exposure scenarios to address potential health effects, and to optimization of pretreatment and countermeasure delivery.

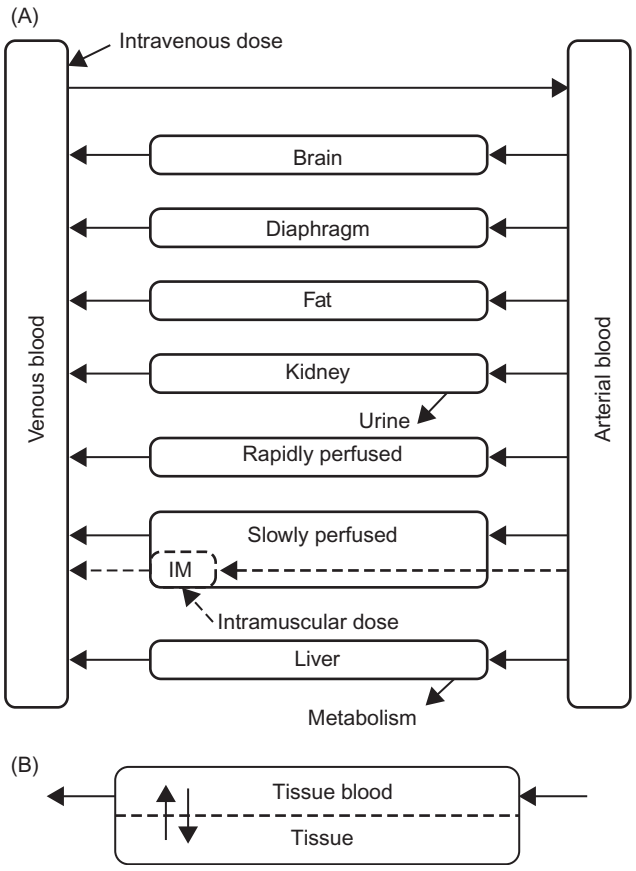

FIGURE 66.1 PBPK model schematic of the distribution of the oxime TMB-4 in rats and humans. Each tissue compartment depicted in schematic A includes diffusion limitation as shown in schematic B (Sterner et al., 2013).

66.7 Experimental and QSAR methodologies to predict blood and tissue partition coefficients

As shown in Fig. 66.1, PBPK modeling utilizes mathematical compartments that conceptually represent the physiology of an organism to depict the pharmacokinetics of chemicals exposed to the body. Traditional PBPK models depend on knowing the partitioning of the agent in key tissues of the organism. The distribution of a chemical within a tissue/compartment is most commonly represented by the following Eq. (66.1) (Krishnan et al., 2001):

$$\frac{dA_t}{dt} = PA\left(CV_t - \frac{C_t}{P_t}\right) \tag{66.1}$$

where A = amount of chemical in the tissue, PA = permeation area cross product for the tissue, CVt = concentration in venous blood emerging from the tissue, Ct = concentration in tissue, and Pt = tissue−blood partition coefficient. Note that $A = Ct \times Vt$, where Vt is the volume of the tissue.

PBPK modeling relies heavily on a proper definition of the partition coefficient because this parameter drives chemical uptake into the tissue. It is generally identified for PBPK modeling as the concentration ratio of the fraction in the tissue to the fraction in the blood at equilibrium (Schmitt, 2008), as shown in Eq. (66.2).

$$P_t = \frac{C_{\text{Tissue}}}{C_{\text{Venous}}} \tag{66.2}$$

The vial equilibration method is the most common in vitro method for determining partition coefficients for volatile or semivolatile materials and has been used most successfully for volatile organic solvents (Gargas et al., 1988). Tissues are harvested from the species of interest and incubated with the test compound until equilibrium is reached between the tissue and the headspace in the vial. The blood/air or tissue/air partition coefficients are given by the ratio of the concentrations of the chemical in the blood or tissue relative to its concentration in the headspace. Tissue−blood partition coefficients are calculated from the respective tissue/air and blood/air values. A number of operational equations have been derived to calculate these ratios under specific experimental conditions. Time to steady state is critical and should be optimized for the test compound. Metabolism of the compound in exposed tissue samples must be controlled. Analysis is performed by gas chromatography in a verified linear range. Human tissues can be obtained from tissue bank organizations to provide species specificity to models developed with human data. To estimate partitions for compounds of low volatility or nonvolatility, the in vitro filtration method of Jepson et al. (1994) can be used. Limitations of all such in vitro methods include issues of how representative the tissue sample is of the often quite heterogeneous tissue in the animal, and how closely the in vitro tissue preparation approximates the in vivo state. In addition, for highly lipophilic compounds, issues related to adsorption of the material onto glass and other surfaces need to be addressed.

Identifying the partition coefficient of a chemical for PBPK modeling is difficult because of the fact that most experimental methods fail to address this proper definition. In vivo methods most commonly track the kinetics of the chemical (radio-labeled or not). However, if enzymes are not properly inhibited, then metabolites can also be tracked instead of the parent compound, leading to contamination of the measured partition coefficient by the more water-soluble metabolite. Experiments are most commonly performed via a single dose at different routes of exposure, rarely leading to the steady state. To reach steady state, a critical point at which measurements should be made, a constant intravenous infusion of a chemical over an extended period of time until the concentration in the blood and tissue becomes constant must

be made. Finally, protein binding is most commonly not accounted for, leading to a total concentration measured instead of the available free fraction. Compounds bound to proteins such as albumin, globulin, and lipoproteins reduce a chemical's tendency to partition into or out of tissues. This can be an extremely important issue depending on the chemical because some are upwards of 90−95% bound in the blood (Poulin and Krishnan, 1996a). These in vivo experimental issues are rarely addressed, which leads to utilization of in vitro measurements where there is greater control of the system.

Because experimental determinations of partition coefficients are slow and expensive, and are fraught with difficulties, particularly for NAs, an alternative is to predict tissue partitioning from more readily available chemical properties (particularly chemical structure), together with key biological properties of the tissue itself, such as its lipid composition, by means of QSAR (Ruark et al., 2008; Sterner et al., 2008). The pharmaceutical industry has utilized QSAR to obtain information of new drug candidates rapidly to help alleviate bottlenecks in the discovery process. A drawback to these models is that they have limited predictive power for scaling across species and to other compounds. However, by utilizing novel mechanistically based methods specifically suited to PBPK modeling, estimations of chemical warfare nerve agent partition coefficient properties can be developed.

Tissue distribution is predicted utilizing a variety of descriptors that range from both chemical-specific to tissue-specific, allowing for universal application of the methods across all species, tissues, and chemicals. A compound's structure and their physiochemical properties, such as the log of the octanol:water distribution (LogP) and the octanol:water distribution mixture of different ionic forms (LogD), are commonly utilized in partition coefficient QSAR, or quantitative structure−property relationships (QSPR) models because they describe the relative affinity for a chemical between hydrophilic and hydrophobic phases. Octanol, being amphiphilic, represents the phospholipid bilayer, which provides a barrier for the cell, separating the extracellular from its intracellular components. However, many studies have shown that octanol is not a perfect surrogate for all lipids within organisms and many are beginning to pursue other surrogates such as vegetable oil and specific phospholipid membranes (Poulin et al., 1999; Schmitt, 2008). Other relevant properties include a wide range of descriptors predicted from ab initio calculations such as H-bonding acidity and basicity parameters, polarity, polarizability, molar refraction, McGowan volume, energies of the lowest unoccupied molecular orbital (E_{lumo}) and the highest occupied molecular orbit (E_{homo}), the maximum positive and negative atomic charge ($\sum Q$), the solvation of free energy (ΔG), as well as many others (Zhang and Zhang, 2006).

As far as tissue characterization is concerned, the fractions of lipid and water tend to be the dominating factors that influence partitioning into or out of the tissue. Some QSPR studies have included proteins that account for an electrostatic interaction with the amino or carboxyl terminal ends of an ionized chemical at physiological pH, as well as some nonspecific binding for neutral compounds. However, a recent QSPR model developed by Schmitt (2008) identified that nonspecific binding to proteins is approximately 40-fold weaker than to lipids. Therefore, proteins may only become of importance for tissues such as muscle and lung that contain a greater fraction of proteins than that of lipids. There have also been attempts at differentiating between phospholipids and neutral lipids (Poulin and Krishnan, 1995a,b, 1996a,b,c; Poulin and Theil, 2000, 2002; Poulin et al., 2001). Rodgers and Rowland (2006, 2007) later differentiated between acidic and neutral phospholipids to account for another electrostatic interaction with ionized chemicals within the tissue. There is also a need to distinguish between tissue and interstitial space, because the interstitial space is highly representative of blood plasma. Other tissue components that may be of interest include DNA, RNA, and intracellular organelles. Binding to DNA appears to be highly correlated with β-blockers; however, the fraction of DNA in tissue is too low to make a substantial difference in the current predictions (Rodgers et al., 2005a,b). Some authors have also suggested differences in intracellular organelle pH as another means of accounting for differences in tissue partitioning for ionized chemicals (MacIntyre and Cutler, 1988; Ishizaki et al.,1996; Daniel and Wojcikowski, 1999; Siebert et al., 2004); however, the overall volume of these organelles in relation to the cytoplasm is very low, most likely not resulting in substantial differences.

Optimization of predictions can be made utilizing linear as well as nonlinear relationships by means of statistical methods to correlate chemical and physiological descriptors to experimental data sets. These statistical methods include multilinear partial least-square analysis, principal component analysis, and neural networking. Many of these tools are included in QSPR/QSAR packages through companies such as Advanced Chemistry Development, SemiChem, EduSoft, BioByte, TOPKAT, MDL, ChemSilico, Pallas, Pharma Algorithms, and others.

A review of the literature will reveal that several QSPR equations have been developed to predict PCs of drugs for use in PBPK models and many are suitable for a wide variety of chemicals. It appears that the greatest deviations in predicted versus experimental measurements of PCs are largely due to an experimental uncertainty or misinterpretations of the data rather than incorrectness of the models. Strategies still need to be developed for other

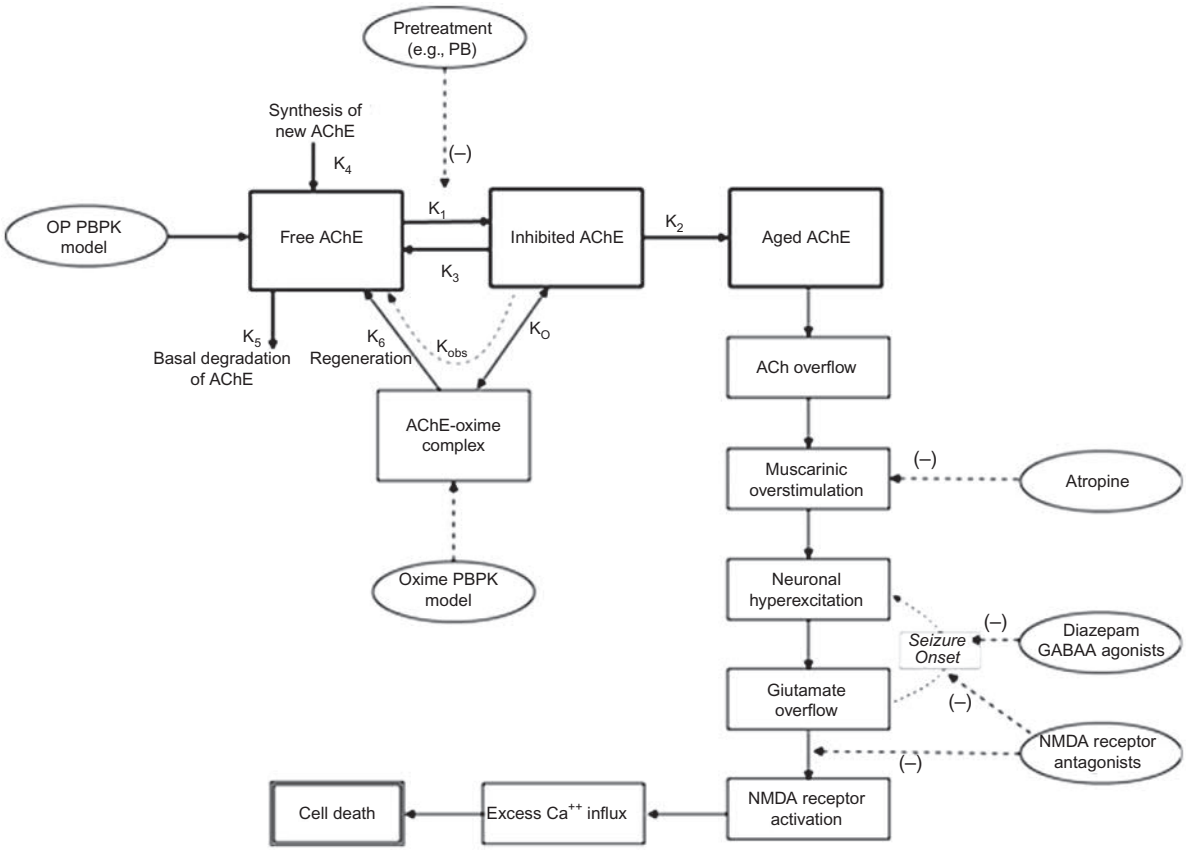

FIGURE 66.2 Schematic for a PD model of AChE inhibition and aging by OPs such as sarin, together with the interaction of pretreatment (pyridostigmine bromide [PB]), and countermeasure treatment (oxime). Pyridostigmine acts as a reversible cholinesterase inhibitor, oxime acts as an AChE regenerator. k_{obs} is an "effective" first-order rate that can be derived as: $k_{obs} = (k_r * [Ox])/(K_D + [Ox])$, when $[Ox] \gg [\text{Inhibited AChE}]$. Potential links for the countermeasures atropine, a muscarinic receptor antagonist, and diazepam, a positive allosteric modulator of the GAB_{AA} receptor and NMDA antagonists, are shown without detailed kinetic interactions.

macromolecular binding (ά-acid glycoprotein) and processes such as blood−brain barrier (BBB) permeation and active transport, but it appears that the methods thus developed are suitable for preliminary PBPK modeling.

66.8 Interaction PBPK/PD model for NAs and countermeasures

As stated, a number of PBPK/PD models have been developed for individual nerve agents (sarin, VX, soman, and cyclosarin) in multiple species. Chapter 58 in the current volume discusses the development of such models. Standalone PBPK or compartmental models have also been developed that describe the pharmacokinetics of certain countermeasures, such as diazepam (Igari et al., 1983; Gueorguieva et al., 2004) and oximes (Stemler et al., 1990; Sterner et al., 2013). However, to date, few models for specific countermeasures have been harmonized and linked to NA PBPK/PD models to be able to quantitatively describe their pharmacokinetic and pharmacodynamic interactions. This is partly due to the fact that most PBPK/PD models developed for NAs and other OPs focus on the inhibition of ChEs as the critical endpoint. The lack of a mathematical description of the disruption of other complex biochemical pathways presents a problem for linking these NA models to those of many countermeasures. For example, the conventional NA countermeasures, atropine and diazepam, as well as many novel countermeasures, do not directly impact ChE kinetics because they act at sites distinct from the active site of the esterases, such as muscarinic, $GABA_A$, or NMDARs (Fig. 66.2).

Interactive models that have been developed and validated exist for countermeasures that compete with NA inhibition of ChE or the regeneration of the free enzyme from its inhibited state, such as pyridostigmine and oximes, respectively. Numerous data in the literature describe the kinetics of interaction between nerve agents (and other OPs) and specific countermeasures at active enzyme sites of concern. For example, Davies and Green (1956) have measured the rate of reactivation of inhibited erythrocyte ChE by various oximes (Table 66.1).

TABLE 66.1 Rate of activation of inhibited erythrocyte ChE by various oximes and hydroxmic acids.

Compound	Inhibitors		
	TEPP	DFP	Sarin
Diisonitrosoacetone	8.4	0.8	24.3
Monoisonitrosoacetone	6.8	0.7	22.1
*iso*Nitrosoacetophenone	10.7	5.1	4.1
*iso*Nitrosoacetylacetone	0.7	NA	1.1
Picolinohydroxamic acid	2.9	0.6	0.2
Micotinohydroxamic acid metabolide	0.3	0.05	0.3

Rates of reactivation are given in L/mol/min.: temp. = 25°C, pH = 7.4.
NA, not available.
Source: Davies, D.R., Green, A.L., 1956. The kinetics of reactivation, by oximes, of cholinesterase inhibited by organophosphorus compounds. Biochem. J. 63, 529–535.

TABLE 66.2 Rate constants for interactions of AChE, OPs and oximes.

		Reactivation constant		
Inhibitor	Oxime	*kr* (min^{-1})	K_D (μM)	[Oxime] (mM)
MFPCh	Obidoxime	0.020	1133	0.5–4
	2-PAM	0.004	2949	1–5
	HI 6	0.076	1233	0.3–2
	HLö 7	0.051	606	0.1–4
MFPβCh	Obidoxime	0.015	1658	0.5–4
	2-PAM	0.002	3131	0.5–10
	HI 6	0.090	859	0.5–4
	HLö 7	0.078	458	0.5–4
MFPhCh	Obidoxime	0.009	3547	1–5
	2-PAM	0.003	3837	1–5
	HI 6	0.028	1037	0.5–4
	HLö 7	0.011	599	0.3–3

Source: Modified from Worek, F., Thiermann, H., Szinicz, L., 2004. Reactivation and aging kinetics of human acetylcholinesterase inhibited by organophosphonylcholines. Arch. Toxicol. 78, 212–217.

More recently, Worek et al. (2004) determined kinetic rate constants of inhibition, reactivation, and aging for different NAs, pesticides, and oximes with human erythrocyte AChE (see Fig. 66.2 and Table 66.2), as described by the reactions listed here:

$$[E] + [OP] \rightarrow^{k1} [EP]$$
$$[EP] + H_2O \rightarrow^{k2} [EA]$$
$$[EP] + H_2O \rightarrow^{k3} [E] + [OP]$$
$$[EP] + [OX] \leftrightarrow^{kD} [EPOX] k_D \rightarrow^{kr} [E] k_r + [POX]$$

where [E] is the active enzyme (i.e., AChE), [OP] is the organophosphorus compound, [EP] is the reversibly phosphylated AChE, [EPOX] is the Michaelis–Menten-type phosphyl-AChE-oxime complex [EPOX], [OX] is the oxime, [POX] is the phosphylated oxime, and [EA] is the dealkylated, "aged" AChE. The reaction rates used describe the bimolecular inhibition (*k1*), aging (*k2*), spontaneous (*k3*), and oxime-induced reactivation. The dissociation rate, K_D, is equal to the ratio [EP] × [OX]/[EPOX] and (*kr*) represents a first-order rate constant for the displacement of the phosphyl residue from [EPOX], resulting in regenerated enzyme and a phosphorylated oxime.

Hence, both a high affinity of the oxime to the "inhibited" or phosphylated AChE and a high regeneration rate (*kr*) are critical for oxime efficacy. Using in vitro enzyme kinetic constants and in vivo inhibitor and oxime concentrations, Worek et al. (2005) were able to extrapolate the percent of AChE inhibition in humans after sarin or cyclosarin (intravenous) and a simultaneous intramuscular injection of various oximes. Similarly, kinetic models have been developed to predict a dose range of human butyrylcholinesterase (HuBChE) required to maintain an adequate residual RBC AChE level after exposure to sarin, soman, or VX (Ashani and Pistinner, 2004). These and other data sets can be readily integrated into the full PBPK/PD modeling framework, as shown in Figs. 66.1 and 66.2. For example, Fig. 66.3 shows a simulation of RBC AChE inhibition and regeneration in the Rhesus monkey after exposure to sarin, followed by 2-PAM administered at 9 minutes after sarin and produced using the PBPK/PD model for sarin presented in Chapter 58. The lower simulation represents AChE inhibition after sarin exposure alone, whereas the upper simulation was generated by fitting an effective first-order rate constant $\left(k_3^{\text{eff}}\right)$, which can be derived as shown in Eqs. (66.3) and (66.4). Improved simulation of the early time points of AChE regeneration may be achieved through linking the sarin model with a PBPK for 2-PAM, which would capture the distribution phase of the countermeasure as well taking into account its affinity.

$$k_{\text{obs}} = \frac{k_r^*[Ox]}{K_D + [Ox]} \tag{66.3}$$

$$k_3^{\text{eff}} = k_3 + k_{\text{obs}} \tag{66.4}$$

Specific model features relevant to the agent's route of exposure and its biological effect can be incorporated when appropriate, for example, quantitative models for ciliary removal of NAs in the upper respiratory tract

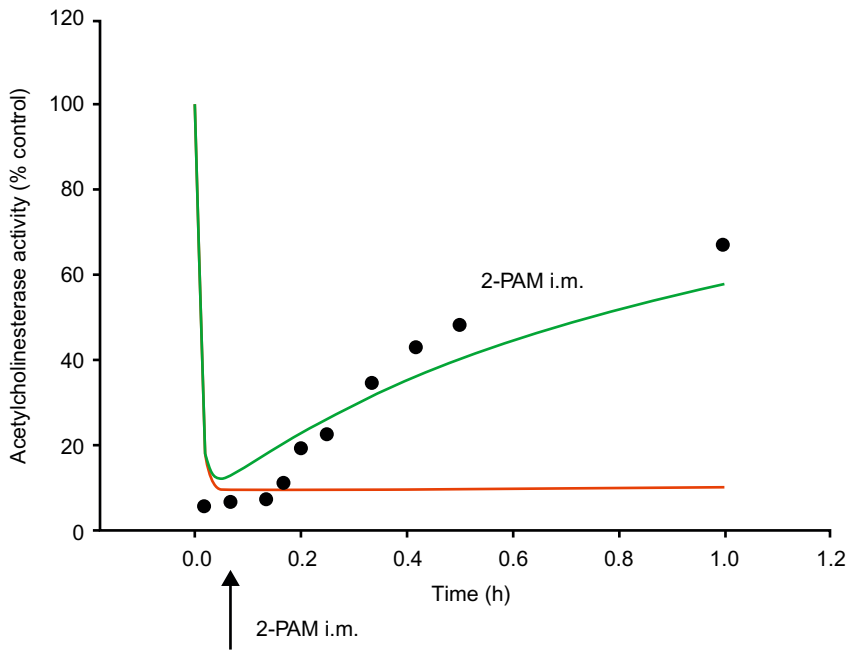

FIGURE 66.3 RBC AChE inhibition in Rhesus monkey after administration of sarin 0.75 LD_{50} (15 μg/kg) intravenous, and with 2-PAM (25.8 mg/kg) administered intramuscularly at 9 minutes after sarin intravenous administration. Atropine was administered (0.4 mg/kg) intramuscularly 15 minutes before sarin administration. The filled circles indicate experimental data (Woodard et al., 1994); the curves show our PBPK model simulations of AChE activity after sarin, both with (upper curve) and without (lower curve) 2-PAM administration.

during inhalation exposure. In addition, it is important to take into account (and incorporate into the model) the limitations of the analytical methods used to quantify circulating and tissue levels of NA, such as the regeneration of NA from its binding sites. For example, it is desirable to model competition for AChE binding sites at erythrocytes and at target sites in the brain; the former because this regenerated NA from RBCs can be interpreted to calculate a measure NA systemic exposure and the latter, of course, because of the health impact. In general, the PBPK models can be linked via interaction processes at target sites.

Pharmacokinetic/PD models have been developed and used to predict the pharmacological effect of seizure activity for several anticonvulsants for which drug receptor kinetics were available from either in vitro or ex vivo binding studies. Diazepam, an agonist of the $GABA_A$ receptor, is among the NA antidotes, for which pharmacodynamic impact on electroencephalogram (EEG) activity has been modeled. Dunhof et al. (2007) have developed compartmental kinetic models for various anticonvulsants and modeled their ligand receptor pharmacodynamics. EEG parameters have been used as pharmacodynamic endpoints (Tuk et al., 1999; Van der Graaf et al., 1999; Bueters et al., 2003; Dunhof et al., 2007). These receptor binding models predict changes in receptor binding and pharmacodynamics as a function of changes in the pharmacokinetics of the ligand as described in Eq. (66.5):

$$E(C) = E_{\mathrm{maxA}} \cdot \left(\frac{C_{\mathrm{A}}^{\mathrm{N}}}{K_{\mathrm{A}} + C_{\mathrm{A}}} \right) \tag{66.5}$$

When $E(C)$ is the observed effect at concentration C, E_{max} is the maximal effect (based on EEG components), K is the concentration at half-maximal effect, and N is the Hill factor, a constant expressing the sigmoidicity of the concentration−effect relationship. Subscripts A refer to the specific ligand of a ligand-gated ionotropic receptor.

Unfortunately, the impact of anticonvulsants on NA-induced seizures has not been modeled in this way. Given that many countermeasures have different ligand receptor interactions, such an approach would need to be expanded to the other excitatory and inhibitory neurotransmitters and their respective receptors, which are involved in seizure induction and sustainment. However, validation of such an approach, using components of gross EEG recordings, may not be plausible because of the large number of ionotropic channels represented. An endpoint such as sustained elevated glutamate may be more appropriate.

In addition, many target receptors undergo rapid changes in their level of expression during status epilepticus. $GABA_A$ receptors are undergoing rapid internalization, whereas AMPA and NMDARs are increasing at excitatory synapses, aggravating the loss of inhibitory tone (Naylor et al., 2005; Naylor, 2010). Fig. 66.4 illustrates a basic model of $GABA_A$ receptor trafficking that can be developed into a full PBPK/PD model to include time-critical changes in target receptor activity (Merrill, 2012), leading to benzodiazepine pharmacoresistance. Such a model could be expanded to include other therapeutic targets, such as the NMDAR; when linked with

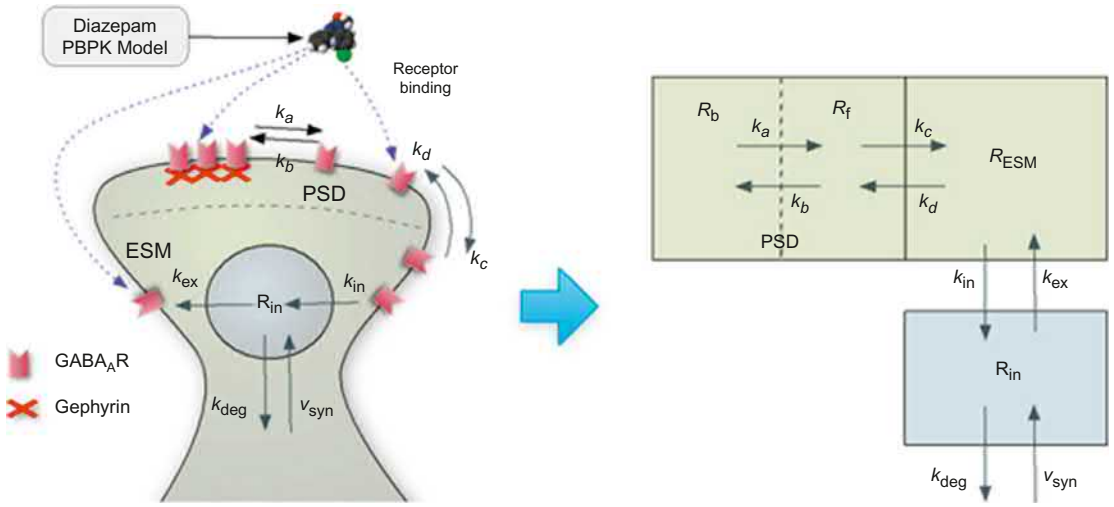

FIGURE 66.4 Simplified model of GABA$_A$R trafficking. *Rb* and *Rf* represent surface GABA$_A$*Rs* within the PSD that are either bound to scaffolding proteins or free, respectively. R_{ESM} represents extrasynaptic GABA$_A$Rs and R_{in} represent internalized receptors. First-order rates are used to describe binding (*kb*) and unbinding (*ka*) of receptors to scaffolding proteins within the PSD, lateral movement between the synaptic and extrasynaptic membrane (*kc* and *kd*), internalization (k_{in}), recycling and membrane insertion (k_{ex}), and degradation (k_{deg}). Synthesis of nascent receptors is described with a zero-order rate, V_{syn}. *Adapted from Merrill, E., 2012. A Mechanism-Based Model to Describe GABAA Receptor Trafficking and Benzodiazepine Pharmacoresistance during Status Epilepticus. (Electronic Dissertation). Retrieved from: <https://etd.ohiolink.edu/>.*

PBPK models for the corresponding therapeutics, they can be used to optimize dosing based on the time from onset of status epilepticus.

Although the receptor targets of various NA countermeasures vary distinctly, be they ion channel-linked, G-protein-linked or enzyme-linked, their pharmacodynamic effects often converge on the final steps leading to the disruption of intracellular Ca^{2+}, beyond which cell death is inevitable. Hence, ultimately, a systems biology approach may provide the best predictions of countermeasure efficacy, especially for modeling long-term low-level effects. The Systems Biology Markup Language (SBML) project is an effort to create a machine-readable format for representing computational models in biology. SBML provides an input and output format, so that different software tools can operate on the same representation of a model, removing chances for errors in translation. SBML also provides convenient model databases, such as Biomodels (www.biomodels.net) for sharing these models. Neural Open Markup Language (NeuroML) similarly facilitates building, simulating, testing, and publishing of models describing channels, neurons, and networks of neurons. Future integration, via a common computational language, of these molecular and cellular level models with PBPK models will permit the development of multiscale models that will link external exposure and tissue dosimetry with NA-induced neurotoxicity. This multiscale approach is necessary for quantitative assessments of the health risks associated with NA exposure.

66.9 Health effects assessment and countermeasure optimization

Once validated, PBPK/PD models can be used to predict efficacy of specific countermeasures, countermeasure combinations, and dosing regimens for specific NAs and combinations thereof. To do so, appropriate measures of efficacy need to be defined. Perhaps the simplest way to estimate the effectiveness of a particular countermeasure is to determine the degree of a receptor activation *at the target site of concern* (e.g., brain or brain region) at a certain time or period of time after the administration of the countermeasure(s), compared with the situation in which no countermeasure is administered. Expressed as a simple ratio, countermeasure effectiveness (CE) may be written in terms of the time-weighted average concentration (from time $t = T_1$ to $t = T_2$) of receptor occupancy (obtained from the PBPK/PD model):

$$\mathrm{CE} = \frac{\int_{T_1}^{T_2} [E]_c \mathrm{d}t}{\int_{T_1}^{T_2} [E] \mathrm{d}t}$$

Here, the numerator is calculated by running the model in the presence of the countermeasure, whereas the numerator is obtained in the absence of countermeasures. The ratio CE can be calculated under a variety of countermeasure dosing regimens to determine an optimum for each specific NA exposure scenario. Clearly, if such an exploration of countermeasure effectiveness were to be conducted experimentally, without the benefit of

modeling, then it would be prohibitively expensive and time-consuming, thus demonstrating the usefulness of the modeling. The information obtained in this way would provide a rational basis for designing countermeasure delivery systems for optimized effectiveness. Ultimately, such an approach would also aid in the design of novel countermeasures, either to be used alone or to be integrated into an optimized countermeasure delivery "package."

A separate, although related, application of the proposed modeling approach is the development of meaningful therapeutic animal models based on rational animal to human scale-up. In such an application, dosing regimens for existing countermeasures and countermeasure combinations, and particularly novel countermeasures, can be tested in experimental animals with the knowledge that the results obtained can be extrapolated to humans by taking into account the appropriate species differences incorporated into the models.

66.10 Concluding remarks and future directions

It can be expected that, in the near future, PBPK/PD models will be developed that can predict the interaction between mixtures of NAs and countermeasures and their opposing pharmacodynamic effects. Validation of such complex modeling efforts is data-intensive and will rely on compilation of existing in vitro, in vivo, and ex vivo data. Unfortunately, the majority of toxicological studies of NAs measure lethality, with little or no additional collection of time-course data of pharmacodynamic endpoints. Similarly, interaction studies between NAs and antidotes often measure only efficacy in terms of a reduction in cell death. This is understandable because of the difficulty and cost in measuring these NAs in biological matrixes and the large number of animals involved in collecting time-course data. Measurements of neurotransmitter changes after OP and countermeasure exposures are very difficult, and often there is uncertainty in deciding which biomarker is most predictive of an effect that is not easily quantifiable, such as cognitive deficits. PK/PD analyses may not always be possible if the relationships between the input (the concentrations of the NA and countermeasure) and the output become too indirect. However, it should always be remembered that even without formal concentration–effect analyses, there is always much to be gained if a pharmacological countermeasure test is performed with intensive sampling of PK and PD parameters. Differences in PK/PD parameters give quantitative information about different neurotransmitter systems in the central and peripheral nervous systems, which cannot be distinguished by pure statistical group comparisons. PK/PD analyses largely eliminate pharmacokinetic variability and provide detailed and functional characteristics of pharmacological systems in the brain or other target organs.

With specific regard to finding novel therapeutics for preventing or ameliorating NA-induced brain damage, PBPK/PD modeling provides a means to extrapolate experimental findings in animals to humans. Differences seen in the efficacy of different therapeutics may sometimes be explained by pharmacokinetics. Perhaps the therapeutic dose reaching a critical tissue, such as the diaphragm, heart, or brainstem is too little, too late, or not long enough. In addition, many of the animal toxicity studies performed to assess the efficacy of a therapeutic against NA measure a biomarker, such as cell death within 24 hours from NA exposure. Biomarkers measured at 24 hours may not always be indicative of outcome. Whether any protection seen at that point would still remain if the biomarker (e.g., cell death) were measured days, weeks, or months later then remains unknown. For example, damage seen in thalamus at 24 hours might later extend to areas with which it has strong reciprocal connections, such as the cerebral cortex. Finally, in the quest for antidotes that may rescue one from cognitive deficits later, when realistically administered beyond the time when the glutamatergic phase takes control of seizure activity (i.e., >40 minutes after NA exposure), predicting biomarkers that indicate the promotion of physiological balance in the brain, rather than the attenuation of seizure activity, may become the most important endpoints for predicting the efficacy of novel countermeasures.

Given the unsustainable cost and time involved in bringing novel therapeutics to market, the use of quantitative techniques to improve countermeasure development are being used increasingly more. Several opportunities exist in drug development, which could lead us on a more direct path toward finding suitable countermeasures, for example, more complete integration of the available knowledge of therapeutic candidates early in their development using PBPK/PD model(s) for NAs and countermeasures together. This should be started early, in the preclinical phases of drug development. Extrapolation of animal PBPK/PD models to humans for simulating initial clinical trials should be preformed to evaluate the design of proposed clinical trials of a drug development plan, and to provide proof of concept. This ability to mathematically explore various exposure–response relationships of NA(s) and countermeasure(s) is useful for designing optimal countermeasure regimens and identifying the limits of the therapeutic window after NA exposure. Uncertainty analysis of the parameters of the model may identify biomarkers that may be critical in describing the data. Ways of defining and decreasing the uncertainty in efficacy and safety (risk assessment) in the drug and disease model are needed.

Finally, the mechanisms (e.g., diffusion-limited transport, receptor binding, protein upregulation or desensitization) described in a particular NA countermeasure model should be updated as new information is generated, thereby using the models as knowledge management tools. Thus, modeling the PK/PD of neuroprotectants in the presence of NA may provide direction and guidance early during clinical development, when there is opportunity to change direction and plans.

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Chapter 67

Research on medical countermeasures for chemical attacks on civilians

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67.1 Introduction

Chemical warfare agents (CWAs) are highly toxic chemicals that have been used in military conflicts, beginning in World War I and continuing in more recent conflicts in Syria (Sellström et al., 2013) and during the Iran−Iraq War in the 1980s (Haines and Fox, 2014). Since the 2001 terrorist attacks in the United States, there has been an increased effort to better prepare for the potential deployment of biological, radiation/nuclear, and chemical weapons against civilian targets. Underscoring these concerns are events surrounding the use of the CWAs sarin and *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methyl phosphonothioate (VX) by the terrorist organization Aum Shinrikyo against Japanese civilians in the 1990s (Vale, 2005; Yanagisawa et al., 2006). These concerns were reinforced when sarin was deployed against civilians in the suburbs of Damascus, Syria, in 2013, during the Syrian civil war (Dolgin, 2013). The US Department of Health and Human Services (HHS) and other federal agencies have developed and sustained a highly focused effort to assess, and if necessary, improve current emergency response capabilities in the event of a terrorist event. These efforts include both preventative capabilities such as personal protective equipment (PPE) for first responders who must enter a contaminated site, and medical response capabilities, such as safe and effective treatments and diagnostic tools to reduce mortality and morbidity after a chemical attack.

Medical countermeasures (MCMs) for mass casualty chemical events are also important for large-scale industrial accidents and general poisonings (Table 67.1). The 1984 Union Carbide industrial accident in Bhopal, India, released 40 metric tons of methyl isocyanate and resulted in over 3000 deaths. Transportation of toxic chemical can often lead to accidents such as the train derailments and leakage of chlorine (Fig. 67.1). Additionally, there is an exceedingly large number of general chemical poisonings from nonoccupational exposures. The Poison Control Centers in the United States receive millions of calls each year due to unintentional human exposures and poisonings. Taken together, chemical exposures from warfare, terrorism, industrial accidents, and general poisoning account for a large burden of illness and the availability of safe and effective medical treatments for these toxic exposures is a global unmet need.

CWAs can generally be divided into four broad categories: traditional nerve agents, such as sarin and VX; vesicating agents, such as sulfur mustard; pulmonary agents, such as chlorine and phosgene; and metabolic poisons, such as cyanide. Detailed descriptions of medical interventions for each of these CWAs can be found elsewhere in this book. The civilian chemical threat spectrum includes CWAs, toxic industrial chemicals (TICs), such as hydrogen sulfide, pesticides, such as parathion and brodifacoum, and pharmaceutical-based agents such as opioids. There are many other agents that pose a threat to civilians because of their toxicity and availability. Antidotes that are specific to a chemical will need to be developed, however, many of these chemicals can be grouped based on acute effects and pathologies they have in common, so that the therapeutics being developed will have a broader spectrum of activity, that is, efficacious

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TABLE 67.1 Mass casualty chemical events.

	Examples
Chemical warfare	• World War I and II: thousands of fatalities • Iran–Iraq War (1980–88): thousands of fatalities • Current conflicts in the Middle East: unknown
Terrorism/nonmilitary malicious use	• Tokyo Subway Attacks (1995): thousands affected; 13 fatalities • Jonestown mass suicide (1978): 900 fatalities (TimeInc., 1979) • Tylenol and Excedrin poisonings (1980s): few fatalities
Industrial accidents	• Common occurrence; thousands of injuries and fatalities annually • Bhopal Union Carbide disaster (1984): 5000 fatalities
General poisonings	• Over 2 million calls to Poison Control Centers for human poison exposures each year (Poison Statistics National Data, 2016)

FIGURE 67.1 Train wreck and chlorine spill in Graniteville, SC, in 2005. *Accessed from www.cen.online.org, June 23, 2008.*

against more than one chemical threat. Chemical toxidromes have been developed (Ciottone, 2018) which include acetylcholinesterase (AChE) inhibitors, asphyxiants, anesthetics, caustic agents, blood agents, pulmonary agents, and opioids. Several antidotes under study (see below) show promise as being effective for more than one chemical threat.

67.2 Medical countermeasures used in civilian chemical incidents

Medical interventions must be appropriate for a diverse civilian population, and treatment strategies must be based on the toxicokinetics and pharmacokinetics of the chemical threat and the therapeutic agents, respectively. Most, but not all, chemical threat agents are fast-acting lethal poisons that lead to rapid cardiopulmonary arrest. However, exposure to these agents during a mass casualty event would likely lead to an exposure level gradient ranging from lethal doses close to the point(s) of release, to low-level exposures that are not toxic at all. The resultant opportunity for effective medical intervention is among those individuals that survive the immediate lethal effects until first responders arrive, and those who receive sublethal exposures but still need medical attention. These opportunities will depend on the route of chemical threat exposure and the method of drug delivery. How a toxic chemical enters the body often determines the time window for possible medical intervention, which is often very short. Thus, treatments must be administered easily and rapidly in a situation involving mass casualties. Both immediate and long-term effects of exposure to chemicals must be understood to ascertain whether treatments are needed and whether they can be developed. Drugs should be chemically and physically stable so that they are amenable to prepositioning and stockpiling, and pretreatments for first responders to a contaminated site should be considered, especially when decontamination is impossible.

If the action at the target site of the chemical agent does not cause immediate death, there are several strategies that can be employed to reduce the impending morbidity and lethal effects of chemical threat agents. While many of the effects will be treated symptomatically to restore cardiopulmonary function or other effects such as chemical burns and ocular damage, there are some specific treatments for nerve agents and cyanide available. Most nerve agents of concern are organophosphorus (OP) CWAs such as sarin, VX, tabun, and soman, or OP pesticides like parathion and chlorpyrifos. These agents inhibit AChE and cause an excess of the neurotransmitter acetylcholine (ACh) that causes overstimulation of cholinergic receptors. For OP nerve agents and OP pesticides, acute poisonings are treated with atropine sulfate to inhibit peripheral and central muscarinic effects, and pralidoxime chloride (2-PAM) to reactivate inhibited AChE. Atropine

blocks muscarinic cholinergic receptors in the parasympathetic nervous system to reduce excessive secretions and smooth muscle contraction (Jett and Spriggs, 2018). It does not have a significant therapeutic effect for central nervous system (CNS) toxicity, nor does it appear to work well at cholinergic synapses on skeletal muscle. Prolonged seizure activity (status epilepticus) after exposure to OPs is controlled by the benzodiazepine anticonvulsant diazepam, or the recently FDA-approved similar drug, midazolam (Seizalam).

There are two FDA-approved cyanide antidotes currently available: Cyanokit (hydroxo-cobalamin) and Nithiodote (sodium nitrite and sodium thiosulfate). Additionally, there is a third treatment, amyl nitrite, which has traditionally been packaged with sodium nitrite and sodium thiosulfate as the Cyanide Antidote Kit. Cyanokit and Nithiodote require intravenous administration by trained personnel. While these antidotes are effective for single cyanide poisonings, better antidotes for mass casualty events are a current unmet need. The first medical product in the United States for use on certain injuries caused by sulfur mustard was cleared recently. The product, Silverlon, can be appropriate for use on first- and second-degree skin burns caused by exposure to sulfur mustard. While there are no FDA-approved drugs for chlorine and other pulmonary agents, research is ongoing with several promising therapeutics. There are no other treatments specifically for other chemical threats, including the many toxic industrial and agricultural chemicals, as well as pharmaceutical-based agents that could be used in mass casualty civilian events.

Military research on the effects of the OP nerve agents and mustard gases has been ongoing for several decades since their discovery and use in past wars. This work, along with efforts in the nonmilitary research community, has led to the development of useful therapeutics and diagnostic tools primarily for use to protect soldiers on the battlefield. Of particular note are the development of more effective PPE, prophylactic drugs such as pyridostigmine bromide (PB) that reversibly binds to AChE (Haigh et al., 2005), and portable antidote kits like the MARK-1, Antidote Treatment Nerve Agent Autoinjector (ATNAA), and Duodote autoinjectors (Henretig et al., 2002). While there is mostly overlap in military and civilian research, there are some key differences. First, the typical soldier is fit and healthy and between the ages of 18−45, whereas the civilian demographic is much broader, including pediatric and elderly people, pregnant women, and individuals with preexisting medical conditions such as asthma, heart disease, and diabetes. These factors are extremely important when deciding the specific type and dosage of treatments that can be administered during a civilian chemical emergency. Fortunately, some military countermeasures can be adapted to special populations such as children (Baker, 2007; Rotenberg and Newmark, 2003). Second, there is a greater need for pretreatments for soldiers who enter into conflicts where chemical weapons are a possible threat. For the purpose of this chapter, we define pretreatments as those medical countermeasures to be administered before exposure. For both civilian and military events, personnel who would be pretreated with medical countermeasures include first responders, such as emergency medical technicians and persons responsible for site decontamination.

Emergency department medical personnel, who are likely to treat individuals shortly after a chemical incident, may also require pretreatment to prevent incapacitation from exposure to residue and off-gassing of CWAs from patients. These secondary exposures could be deadly and lead to more loss of life, both during and after an event. In theory, prophylactic drugs would be administered in conjunction with proper PPE to first responders before they enter a "hot zone." However, depending on the possible adverse effects and pharmacokinetics of the proposed drugs, there may be some operational difficulties in the implementation of a prophylaxis program. For example, compliance by otherwise healthy individuals may present an issue if there are potential side effects. These potential side effects that may occur when a countermeasure is administered to otherwise normal and unexposed individuals illustrate the importance of favorable safety profiles, especially if the intended indication is for prophylaxis use. Side effects in first responders and care providers could compromise their ability to respond effectively to chemical events involving civilians or to execute the military mission. The only pretreatment currently approved by the FDA for nerve agents is PB, which was first approved for treating myasthenia gravis and is now approved for treating intoxication by soman only. It is to be used by military troops at high risk of exposure to soman on the battlefield. PB is a reversible inhibitor of AChE and protects it from permanent inhibition by soman.

67.3 Research needs for civilian medical countermeasures

The current standard of care for nerve agents is effective, but there are some areas for improvement. For nerve agents, administration of 2-PAM will succeed only if irreversible covalent modification of the nerve agent + AChE complex has not occurred (a process called aging). Unaged AChE enzyme is thus restored to the active state and can continue the hydrolysis of ACh. If the enzyme complex is aged, then 2-PAM is totally ineffective and the nerve agent soman is a good example where rapid aging prevents 2-PAM from being useful. There is a need

to find better AChE reactivators, and those that have better penetrance of the blood–brain barrier to reverse the seizures associated with these compounds. Atropine is effective for blocking the overstimulation of cholinergic receptors in the periphery but less so in the CNS. Centrally acting cholinergic receptor antagonists are needed that have a favorable distribution in the CNS. Anticonvulsants such as midazolam and diazepam are used to prevent or treat seizures, but seizures can become refractory to these treatments and spontaneous recurring seizures often occur (Guignet et al., 2019). Whether there is sufficient time after an exposure to the CWA for a therapy to be beneficial depends on the initial effective dose. This determination is based on several factors, including the initial level of the agent released, proximity to the point of release, route of exposure, and duration of exposure. If the effective cumulative dose is low, the risk of an acute, life-threatening intoxication will likely not be an issue, but there is significant evidence that acute sublethal exposures to chemical nerve agents may be associated with long-term neurological sequelae. For example, the US National Toxicology Program concludes that acute sarin exposure is "known to be a neurological hazard to humans" based on a comprehensive unbiased systematic review of the long-term effects of sarin (NTP, 2019). These gaps in the overall treatment of OP nerve agents and pesticides are topics of current research.

Victims exposed to a sublethal concentration of the fast-acting poison cyanide are treated with ventilation and supportive care to delay mortality and morbidity until they can be taken to medical facilities. New and improved antidotes should have the following essential characteristics: (1) they can be administered by minimally trained individuals, possibly with intramuscular autoinjectors, oral, intranasal, or inhalational administration, (2) have a rapid onset and long duration of efficacy, (3) efficacy for all forms of cyanide and routes of cyanide exposure, (4) have a ready-to-use antidote not requiring preparation in the field, (5) stable for storage of many doses, and (6) be cost effective. Current research on better cyanide antidotes includes basic mechanistic research to discover targets, through preparing candidate therapeutics for advanced development and ultimate FDA approval and licensure. Cyanide research can be categorized as either cyanide-based or host-based. Cyanide-based research includes agents that bind cyanide and remove it from circulation, while host-based research focuses on the detoxification of cyanide by increasing its metabolism and elimination, or by direct interaction with targets of cyanide and other mechanisms that prevent or ameliorate acute and chronic toxicity. Special consideration is being given to alternate routes of administration and/or dosing regimens for new or already FDA-approved therapies that would be safer, more effective, or easier to administer during a mass casualty scenario. Specific subpopulations (e.g., pediatric and pregnant) that are more vulnerable are also a priority for civilian exposures. Finally, clinical efficacy studies are rare because of the obvious ethical challenges, however some studies may be possible in people who are exposed to cyanide accidentally, for example from smoke inhalation.

Another important area of research is to identify new approaches to mitigate the pulmonary edema that typically develops after lung injury caused by agents such as chlorine and phosgene. Some agents, such as chlorine and sulfur mustard, may also cause delayed toxicity after initial exposure or long-term pulmonary effects (such as fibrosis) years later (Wolfe et al., 2019). Therefore, medical interventions aimed at improving the long-term health outcomes of nonlethal exposures to pulmonary agents are also critical. Since the immune responses observed in the pulmonary system after sulfur mustard or TIC exposure share similar characteristics, it is likely that therapeutic drugs that prevent, slow, or halt the processes of inflammation and subsequent cellular damage are desired. While sulfur mustard is rarely fatal after acute exposure, treatments that reduce vesicant damage to the skin and initial injury to the lung are a priority.

The last area of research needed is basic mechanistic research with the many emerging chemical threats to civilians, including TICs and pharmaceutical-based agents. For many of the TICs, little is known about their toxicity mode of action and potential targets for therapeutic intervention. The need for good natural history models is important for determining how and when to administer potential treatments. The opioid crisis and the availability of these highly toxic drugs calls for research on MCMs that would be appropriate if they were used in deliberate attacks or accidental releases resulting in mass casualties. The research might include industrial accidents, natural disasters causing casualties, or deliberate attacks against civilians. Examples of research needs include safety and efficacy studies of naloxone and other FDA-approved drugs for use against fentanyl and its derivatives, especially as it relates to respiratory depression, and when these opioids are administered by a method consistent with a deliberate attack (e.g., aerosolized, in food or water, etc.). There may be other opioid overdose antidotes with greater safety and efficacy than naloxone and other FDA-approved drugs to match the higher potency of fentanyl analogs. Antidotes with longer half-lives so that multiple doses are not needed in rapid succession are also needed.

67.4 Research at the National Institutes of Health in the United States

The NIH Countermeasures Against Chemical Threats (CounterACT) program is part of the larger NIH Biodefense program and part of the HHS Public Health

Emergency Medical Countermeasures Enterprise (PHEMCE), which coordinates medical countermeasures-related efforts across the Department of Health & Human Services (HHS) and other US Government partners. The overarching goal of the CounterACT program is to integrate cutting-edge research with the latest technological advances in science and medicine to enhance the nation's medical response capabilities during chemical emergencies. This is a trans-NIH effort, involving partnerships with the National Institute of Neurological Disorders and Stroke (NINDS), the National Institute of Environmental Health Sciences (NIEHS), the National Eye Institute (NEI), the National Institute on Drug Abuse (NIDA), the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), the National Heart Lung Blood Institute (NHLBI), and the National Library of Medicine (NLM) to execute the overall NIH Strategic Plan and Research Agenda for Medical Countermeasures Against Chemical Threats. The CounterACT program employs a strategy of collaboration and partnership, it supports research aimed at the discovery and identification of better medical countermeasures, and guides their movement through the regulatory process in cooperation with other federal departments, agencies, and initiatives, such as the Biomedical Advanced Research and Development Authority (HHS BARDA), FDA Medical Countermeasures Initiative (MCMi), and the Defense Threat Reduction Agency (DoD DTRA).

NIH CounterACT has brought together the expertise of researchers in chemical warfare and a vast pool of NIH-funded experts in scientific areas that are relevant to the central questions being posed in the field. For example, if untreated, chemical nerve agents can cause seizures and neuropathological sequelae, conditions that share molecular mechanisms and phenotypes with many neurological illnesses such as epilepsy and stroke. These disorders are research areas that the NIH has supported for decades, and this has provided an unprecedented opportunity for CounterACT to engage researchers and neurologists in these fields. The partnership between HHS and DoD facilitated by CounterACT has led to effective collaborations among scientists who otherwise would rarely interact and produced promising, novel approaches in the development of medical chemical countermeasures. Research within CounterACT has resulted in the transition of several lead compounds to advanced development and FDA approval, as well as numerous publications in some of the most prestigious scientific journals (see www.ninds.nih.gov/counteract).

67.5 Contract core facilities

The overarching NIH Chemical Countermeasures Research Program (CCRP) is a trans-NIH initiative that includes both formal and informal collaborations with the NIH CounterACT program and its participating NIH institutes and centers. The NIH National Institute of Neurological Disorders and Stroke (NINDS) CounterACT program is the lead institute for the program. Oversight of the program is provided by the National Institute of Allergy and Infectious Diseases (NIAID), which manages the Biological Defense Program, the Radiological/Nuclear Program, as well as the Chemical Defense Research Program. The program supports an extensive extramural portfolio of academia, industry, and government research organizations. The portfolio includes research centers of excellence, individual research projects, contracts, support services, and Interagency Agreements (IAAs) with the DHHS and the Department of Defense, specifically the US Army Medical Research Institute of Chemical Defense (USAMRICD), US Army Combat Capabilities Development Command Chemical Biological Center (CCDC), and the Defense Technical Information Center (DTIC).

The NIH Chemical Countermeasures Research Program provides no-cost support services to investigators whose products show promising results as potential medical countermeasures in preliminary testing. These services include the CounterACT Neurotherapeutics Screening Program. This program identifies novel neurotherapeutics that may be administered with approved standard-of-care treatments to more effectively suppress status epilepticus activity and/or mitigate neuropathology after OP agent exposure. The services also include the CounterACT Efficacy Research Facility (CERF), which conducts pilot studies to characterize novel models of lethal and nonlethal effects. The facility obtains independent confirmation of product efficacy in validated small or large animal models of chemically induced toxicity. The CounterACT Preclinical Development Facility (CPDF) conducts non-Good Laboratory Practice (GLP) preclinical studies to characterize and optimize hits identified early in the discovery and development process. Lastly, the CounterACT Ocular Therapeutics Screening (COTS) Program conducts pilot studies to evaluate the efficacy of investigational MCMs against the acute and/or chronic effects of eye exposure to sulfur mustard. The chief purpose of these support services is to assist applicants and researchers in obtaining important proof-of-principle efficacy and/or preclinical data in support of subsequent research applications. An important mission of the NIH Chemical Countermeasures Research Program is to offer guidance to academic, commercial, and government investigators on the use of the NIH Core Facilities, and the grant application process. The NIAID CCRP/NINDS CounterACT program encourages investigators to contact the CCRP and/or CounterACT program staff for advice and guidance on how to advance potential models and therapies

from the initial idea, through the basic research and discovery stages, and forward into the early phases of product development.

67.6 Scope of research

The NIH CounterACT program focuses on basic and translational research on the development of novel therapeutics that will enhance medical response capabilities against chemical threats during an emergency to decrease morbidity and mortality of the civilian population. The emphasis of research is on identifying and optimizing lead compounds that are effective when administered after a chemical exposure has occurred (postexposure efficacy). Countermeasures that are only effective when administered prior to the chemical insult (prophylaxis efficacy) are of lower priority to the program. Therapeutics to prevent long-term or delayed chronic effects after an acute exposure, such as those to be administered after evacuation from the exposure site or during in-hospital care, are also within the purview of the program.

The scope of the research supported by the CounterACT program includes basic research to identify molecular mechanisms of acute toxicity, target and candidate identification and characterization, and lead candidate optimization through the development of appropriate human-relevant models (Fig. 67.2). The primary goal of this effort is to develop models of lethality and serious morbidity consistent with real-life civilian mass casualty scenarios. Using these models, the program supports the generation of in vivo, non-GLP toxicity, pharmacology, and efficacy studies consistent with the product's intended use in humans.

The NIH CounterACT program not only supports the discovery of novel compounds with requisite therapeutic activity and acceptable safety profiles, but also alternate routes of administration for FDA-approved therapeutics that would be safe and more effective to administer during a mass casualty scenario (e.g., intramuscular injection, etc.). Special consideration is given to research that is relevant to populations who are particularly vulnerable, including the young, the elderly, pregnant women, and individuals with preexisting medical conditions. Children and pregnant women, for example, have been shown to be much more sensitive to the toxicity of some CWAs and therefore, may require specialized medical management after exposure (Wright et al., 2016). Consequently, animal models and studies that address these vulnerabilities, as well as long-term effects after an acute exposure are of interest to the program.

67.7 Research on medical countermeasures for civilian chemical threats

Most of the research supported by CounterACT has been with OP CWAs or toxic pesticides. Centrally acting oximes that are more effective after aging of the AChE−OP complex have been the holy grail of OP medical countermeasures research for many years. Also, a big challenge with the use of 2-PAM is that it does not penetrate the blood−brain barrier very well, and its efficacy is controversial (Blumenberg et al., 2018). Novel centrally acting compounds based on substituted phenoxyalkyl oximes show promise in rat models using sarin and VX surrogates (Chambers and Meek, 2019). Reactivation of AChE is a complex problem and translational research in the

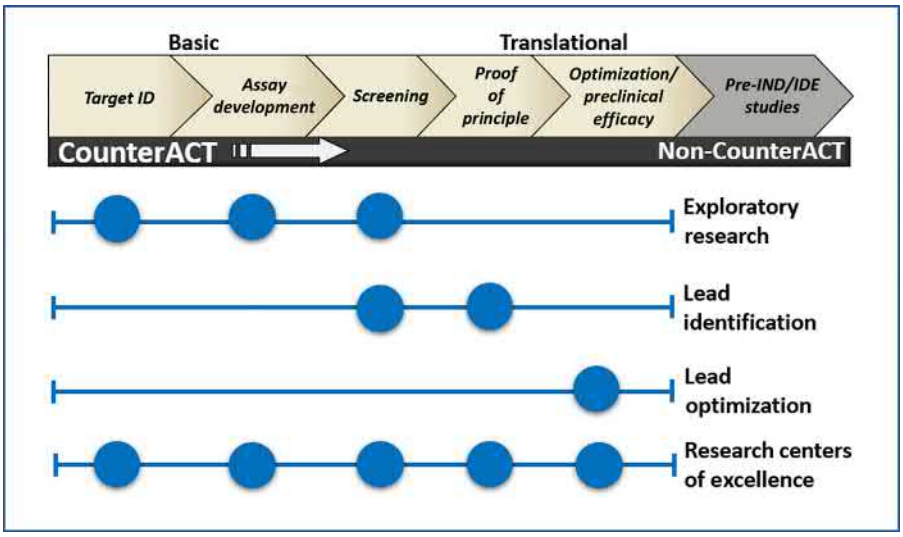

FIGURE 67.2 The NIH CounterACT program funds research across basic and translational science. Basic mechanistic research to identify targets for therapeutic development through lead compound identification and optimization is supported by the various programs. Optimized lead compounds are then poised for advanced development and FDA approval.

neurological research part of the NIH CounterACT program is supported by basic research to help study mechanisms of chemical agent toxicity and how antidotes work. For example, molecular events can now be studied by dynamic imaging in living systems with positron-emission tomography (Thompson and Gerdes, 2019). With regard to seizures, some research suggests that refractory SE caused by OPs may be controlled with a polytherapy approach targeting multiple neurotransmitter systems, rather than midazolam alone (Niquet et al., 2019). Likewise, the glutamate receptor antagonist LY293558 has shown significant promise as a new treatment for recurring seizures and behavioral effects caused by soman, and this is potentiated with the addition of caramiphen (Aroniadou-Anderjaska et al., 2019).

There is also substantial human and animal study evidence that lasting effects may occur in people who survive the acute lethal effects of OP agents (NTP, 2019). Diisopropylfluorophosphate (DFP) is a potent OP pesticide now routinely used as a research tool and it has been shown that the prostaglandin-E2 receptor is a potential target for attenuating DFP-induced hippocampal degeneration when a receptor antagonist is delivered well after status epilepticus has ended (Rojas et al., 2019). Inducible nitric oxide synthase inhibitors prevent some long-term effects after DFP exposure in a rat model and it is a promising follow-on therapy after the standard of care (Putra et al., 2019). Research has revealed that calcium homeostasis, and blockade of calcium release with antagonists ameliorate some of the long-term effects (Deshpande and DeLorenzo, 2019). Neuroinflammation and oxidative stress are also likely involved, and persistent histopathological damage in several brain regions after DFP exposure support this hypothesis (Guignet et al., 2019) and persistent neuroinflammation has been confirmed with in vivo imaging in the rat (Hobson et al., 2019). Oxidative stress brought on by OP-induced seizures may cause some of the long-term effects, but there is also the possibility of direct effects of the OP agents causing oxidative damage, and drug candidates like AEOL 10150 are being looked at as potential general antioxidants that could be used for OP and other chemical threats (Pearson-Smith and Patel, 2019).

Some chemical threats under study cause devastating neurological effects but are not OP compounds. Tetramine or TETS is a GABA-agent convulsant used as a rat poison and causes seizures similar, but not identical to the OP agents. Researchers are developing animal models (Pessah et al., 2016) and testing potential therapeutics that might work for both GABA and cholinergic chemical threats (Vito et al., 2014). The natural history of TETS toxicity is being studied; for example, it is much more potent to juvenile rats than adults (Laukova et al., 2019), and allopregnanolone and ganaxolone are effective in mice for the treatment of TETS-induced SE when administered by the intramuscular route (Zolkowska et al., 2018). Hydrogen sulfide is of major concern, and it has been shown that this compound produces lesions in the brain and the redox agent methylene blue may be an effective antidote (Haouzi et al., 2019).

Much progress on cyanide MCMs has also been made. Cobinamide, sodium tetrathionate, and sodium nitrite with sodium thiosulfate show promise for mass casualty cyanide poisoning. Cobinamide is a vitamin B12 analog that is more potent than hydroxycobalamin as a cyanide antidote, and can be administered by i.m. injection (Ng et al., 2019). It is also an effective antidote against hydrogen sulfide and several other toxic chemicals (Ng et al., 2019). Sodium tetrathionate is a sulfur donor that neutralizes cyanide by converting it to thiocyanate. Like cobinamide, intramuscular sodium rescues >80% of animals in mouse, rabbit, and pig models of cyanide poisoning (Hendry-Hofer et al., 2019). Sodium nitrite and sodium thiosulfate are currently approved for treatment of cyanide poisoning via intravenous injection. It has now been shown that the two drugs can be given by intramuscular injection to rescue animals in the mouse, rabbit, and pig models of cyanide poisoning (Bebarta et al., 2017). Dimethyl trisulfide (DMTS), a sulfur-based molecule present in garlic and onions, is safe and efficacious in rodent models of cyanide injection, ingestion, and inhalation. In lethal rabbit and swine models of cyanide poisoning, DMTS significantly enhances survival and is another promising candidate (Petrikovics et al., 2019). A phenotype-driven screening approach to discover new compound classes for cyanide MCMs has discovered the multivalent cyanide scavenger hexachloroplatinate (HCP) and the metabolic modulator glyoxylate, as potential novel cyanide antidotes (Morningstar et al., 2019). Importantly, both HCP and glyoxylate can be delivered by intramuscular injection. A cobalt complex of the Schiff-base macrocycle 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1] heptadeca-1(17)2,11,13,15-pentaene (CoN4[11.3.1]) is also a viable option as a cyanide antidote (Praekunatham et al., 2019). Ongoing work is investigating the use of CoN4[11.3.1] in combination with a nitric oxide donor to reactivate cytochrome c oxidase, with further improved antidotal capability anticipated. Sulfanegen, a potential antidote for cyanide poisoning with a novel mechanism based on 3-mercaptopyruvate(3-MST) is being developed for the detoxification of cyanide (Patterson et al., 2016). Additionally, sulfanegen can be rapidly administered by intramuscular injection and has shown efficacy in several species of animal models.

Among the most worrisome of pulmonary chemical threat agents is chlorine. Chlorine inhalation can cause severe acute respiratory damage and failure of other organ systems. CounterACT researchers have shown that

oxygen administration can improve survival but may not reduce the probability of respiratory failure and does not improve cardiac and neuromuscular function (Okponyia et al., 2018). Various approaches including the use of nitrite (Honavar et al., 2017) and heparin (Zarogiannis et al., 2014), among others that have been studied as antidotes. Interestingly, chlorinated lipids are formed after exposure to chlorine and could serve as biomarkers and mediators for chlorine gas exposure and toxicity (Ford et al., 2016). Bromine is also being studied and the natural history and mechanism of toxicity is being elucidated so that better MCMs can be developed (Ahmad et al., 2019; Duerr et al., 2018). Another important pulmonary agent being studied is phosgene, which causes debilitating acute lung injury and other effects (Aggarwal et al., 2019). The mechanism of phosphine poisoning is being studied (Wong et al., 2017) and gold(I) complexes are showing some promise as antidotes to phosphine poisoning (Garrett et al., 2019).

Sulfur mustard and other vesicants are being studied at CounterACT Centers of Excellence and in several individual projects. In the lung, this compound causes bronchiolitis obliterans and pulmonary fibrosis (McGraw et al., 2018) and airway tissue plasminogen activator is a promising antidote under advanced development (Veress et al., 2015). A similar compound, nitrogen mustard, also causes pulmonary fibrosis and new insights from researchers indicate that lipid handling and metabolism drive macrophage foam cell formation in the lung (Venosa et al., 2019). In the skin, much is being learned about the mechanism of toxicity (Chang et al., 2018), and new candidate therapeutics are under study (Joseph et al., 2018; McElroy et al., 2016). The vitamin 25(OH)D is a very effective MCM for nitrogen mustard if the dose is timed correctly, or repeat dosing is employed (Das et al., 2018). These studies also led to the development of this treatment for sunburn.

Besides the well-known nerve and pulmonary agents, vesicants, and cyanide, the CounterACT program is working on lesser known agents that are a threat to civilians. Brodifacoum is a long-acting anticoagulant rodenticide that causes severe life-threatening anticoagulant effects. Although vitamin K1 (VK1) treatment effectively prevents mortality, additional methods are needed to reduce the long duration of VK1 treatment, which can last for months at high expense. This compound was used in the adulteration of K-2 synthetic marijuana that led to many illnesses and deaths in the US. CounterACT researchers tested the effects of cholestyramine and found it reduced mortality from 67% to 11% (Lindeblad et al., 2018). Metabolic and other agents like hydrogen sulfide (Ng et al., 2019), other pulmonary agents such as methyl isocyanate (Rancourt et al., 2019), and others are being studied along with a robust and growing portfolio of projects focused on developing MCMs for opioid threats such as fentanyl and its analogs. In this regard the program is focused on the use of opioids that result in mass casualties and require special MCMs suited for treating many victims in a short period of time.

The above discussion only covers a fraction of the literature base on chemical MCMs generated by the NIH CounterACT network of basic, translational, and clinical researchers. The program has also generated over 30 lead compounds poised for, or already transitioned to other agencies for, advanced development and approval by the FDA and inclusion in the Strategic National Stockpile.

67.8 Concluding remarks and future directions

Chemical threat agents have become more of a concern because of their recent use in terrorist incidents, warfare, and individual poisonings. The severe toxicity of these chemicals can be difficult to manage because of their often-rapid modes of action, causing severe injury or death within minutes. The US government continues to support research on improvement and optimization of chemical MCMs. The NIH CounterACT is part of a larger biodefense effort at NIH, it has a dual and complimentary mission: to support basic research focused on understanding chemical toxicities, and to use that knowledge to identify novel targets and develop promising candidate therapeutics for treating exposures that lead to mass casualties. At NIH, a broad research community that otherwise would not be involved in chemical weapons research is being engaged to take advantage of its critical expertise in areas of scientific endeavor relevant to the civilian and military research programs. The NIH program works in concert with the Chemical Medical Countermeasures program at BARDA. BARDA provides an integrated, systematic approach supporting the development and acquisition of the necessary vaccines, treatments, diagnostics, and other medical devices for public health emergencies, including many MCMs emerging from the basic research and preclinical development activities sponsored by NIH CounterACT. For example, NIH and BARDA supported the benzodiazepine drug Midazolam (Seizalam), which was recently approved for a status epilepticus indication and is slated to replace diazepam as a component of the three-drug regimen used to treat nerve agent intoxication. NIH CounterACT will continue basic and translational research on existing and emerging chemical threats, and work to improve preparedness in the United States for chemical emergencies.

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Chapter 68

Pyridinium oximes in the treatment of poisoning with organophosphorus compounds

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68.1 Introduction

Organophosphorus (OPs) compounds have long been used as pesticides and developed into warfare nerve agents such as tabun, soman, sarin, VX, and others. Exposure to even small amounts of an OP compound can be fatal. Death is usually caused by respiratory failure resulting from the paralysis of the diaphragm and intercostal muscles, depression of the brain respiratory center, bronchospasm, and excessive bronchial secretions. The mechanism of OPs poisoning involves phosphorylation of the serine hydroxyl group in the active site of acetylcholinesterase (AChE), which leads to inactivation of this essential enzyme. As a result of AChE inhibition, the accumulation of acetylcholine (ACh) at cholinergic receptor sites produces continuous stimulation of cholinergic fibers throughout the central nervous system (CNS) and peripheral nervous system (PNS), motor convulsions, and epileptic seizures. Accumulating evidence indicates that seizure events are linked to irreversible long-term compromise of cognitive functions and alteration of CNS electrical excitability. Presently, therapy of acute OP poisoning includes administration of atropine as a muscarinic acetylcholine receptor antagonist, together with an oxime, pralidoxime (2-PAM), HI-6, or congeneric *bis*-quaternary pyridinium compounds such as TMB-4 or LüH-6, and CNS depressants such as diazepam. When administered in vivo, quaternary oxime reactivators largely remain in blood and peripheral tissue capable of crossing the blood–brain barrier (BBB) in small amounts and reactivating OP-inhibited brain AChE. This chapter describes the mechanisms of OP action and the role of pyridinium oximes as AChE reactivators in the treatment of OP poisonings.

68.2 Interaction of cholinesterases with organophosphorus inhibitors

The different types of cholinesterases in the human body are characterized by their location in tissues, substrate affinity, and physiological function. The principal ones are acetylcholinesterase (EC 3.1.1.7, AChE), predominantly found in the nervous system and also present in the outer membrane of red blood cells. Plasma cholinesterases (EC 3.1.1.8, ChE), are another group of enzymes present in plasma, liver, cerebrospinal fluid, and glial cells. Under normal physiological conditions, AChE breaks down ACh, which is the chemical mediator responsible for conduction of nerve impulses at the sites of cholinergic transmission. Its physiological role in blood is not understood. On the other hand, ChE is also an esterase that can react in a similar way with most of the compounds that react with AChE. While the effects of inhibition of AChE can be fatal, depending on the dose taken, inhibition of ChE does not have any known consequences on normal body functions (Jokanović and Maksimović, 1997). ChE is a circulating plasma glycoprotein synthesized in the liver, which does not serve any known physiological function. Recent evidence indicates that ChE may have roles in cholinergic neurotransmission, other nervous system functions such as cellular proliferation and neurite growth during the development of the nervous system, and processes in neurodegenerative disorders (Darvesh et al., 2003).

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AChE terminates the action of ACh at the junctions of the various cholinergic nerve endings with their effector organs or postsynaptic sites. In the presence of OP inhibitors, AChE becomes progressively inhibited and is not further capable of hydrolyzing ACh to choline and acetic acid (Jokanović and Maksimović, 1997). Consequently, ACh accumulates at cholinergic receptor sites and produces effects equivalent to excessive stimulation of cholinergic receptors throughout the CNS and PNS.

Both substrate and inhibitors react covalently with the esterases in essentially the same manner due to the fact that acetylation of the serine residue at the AChE catalytic site is analogous to phosphorylation. In contrast to the acetylated enzyme which rapidly separates acetic acid and restores the catalytic site, the phosphorylated enzyme is stable (Fig. 68.1). Inhibited enzyme can be spontaneously reactivated at different rates depending on the OP having branched alkyl groups such as warfare nerve agents and it may occur at a very slow rate.

The variations in the acute toxicity of OPs are the result of their different chemical structures, rates of spontaneous reactivation, and aging. The aged form of phosphorylated AChE is resistant to both spontaneous and oxime-induced reactivation. For example, when inhibited with VX, AChE in red blood cells spontaneously reactivates at a rate of about 1% per hour for about the first 48 h, while the VX-AChE complex ages very little during this period. The soman−AChE complex does not spontaneously reactivate since the half-time for aging is about 2−4 min. The half-time for aging of the sarin−AChE complex is between 5 and 12 h, depending on experimental conditions, and only about 5% of the enzyme undergoes spontaneous reactivation. The half-time for aging of the tabun−AChE complex is about 46 h (Karczmar, 1984; Sidell, 1997; Stojiljković et al., 2001; Milatović and Jokanović, 2009). For OP pesticides containing dimethyl phosphate groups the half-time of spontaneous reactivation of phosphorylated AChE in vitro is about 1 h and the half-time of aging is 3.7 h, while these for diethyl phosphates are slower, being about 31−57 and 31 h, respectively (Worek et al., 1999; Mason et al., 2000; Eyer, 2003).

Although appearing with many phosphorylated AChE complexes, the aging reaction has the major clinical importance and is an imperative problem, particularly in the treatment of soman poisoning. Aging with soman occurs so fast that no clinically relevant spontaneous reactivation of AChE can occur before aging has taken place. Hence, recovery of function depends on the resynthesis of AChE, which is a relatively slow process. After soman exposure, it is important to immediately administer atropine and oximes so that some extent of AChE reactivation occurs before the entire AChE has aged. Even though aging occurs slowly in the case of other warfare nerve agents and OPs, early oxime administration is of high clinical importance for treatment efficiency.

68.3 Clinical aspects of acute organophosphorus poisoning

Signs and symptoms of acute poisoning with OPs are predictable from their biochemical mechanism of action and are directly related to the levels of AChE activity (Table 68.1). The types of effects manifested in these poisonings are muscarinic, nicotinic, and central signs and symptoms. The duration of effects is determined mainly by the properties of the compound: its liposolubility, the stability of the OP−AChE complex, and whether it is reactivatable after the use of cholinesterase reactivators, such as oximes. It is important to note that only OPs containing a P = O bond, known as direct inhibitors, are potent AChE inhibitors; while those having a P = S group, indirect inhibitors, must be metabolically activated to the P = O group (Jokanović, 2001, 2009, 2015). The signs and symptoms of poisoning with direct inhibitors appear quickly during or after exposure, while those with indirect inhibitors appear slower and last longer, even up to several days after cessation of exposure.

Clinical diagnosis is relatively simple and is based on medical history, circumstances of exposure, and the presence of clinical symptoms of poisoning. Confirmation of diagnosis can be made by measurement of red blood cell AChE or plasma ChE. Activities of these enzymes are accepted as biomarkers of exposure and/or toxicity of OPs. Red blood cell AChE is identical to the enzyme present in the target synapses and its levels are assumed to reflect the effects in target organs. Thus, red blood cell AChE is regarded as a biomarker of toxicity of these compounds. AChE in the brain is restored by de novo synthesis more

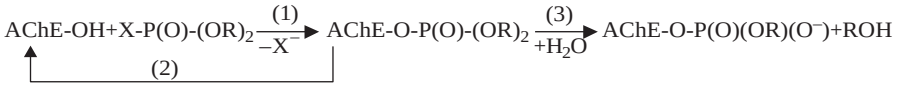

FIGURE 68.1 Interaction of acetylcholinesterase (AChE-OH) with organophosphorus compounds. Reaction 1 shows interaction of the organophosphate molecule with the serine hydroxyl group at the active site of AChE. Inhibited AChE cannot further serve its physiological function, which causes the accumulation of acetylcholine at the nerve endings. Reaction 2 is spontaneous reactivation of inhibited AChE, which occurs very slowly for most OP compounds. Reaction 3, called "aging," represents nonenzymatic time-dependent loss of one alkyl group (R) bound to the phosphorus. The aging reaction depends on the chemical structure of the inhibitor and leads to a stable nonreactivatable form of phosphorylated AChE. X is an acyl radical (i.e., Cl^-, F^-, CN^-, *p*-nitrophenol, etc.) (Jokanović, 2015).

TABLE 68.1 Signs and symptoms of poisoning with organophosphorus compounds (Jokanović, 2015).

Signs and symptoms			Severity of poisoning	Red blood cell AChE (% of control)
Muscarinic	Nicotinic	Central		
Nausea, vomiting, diarrhea, salivation, lacrimation, bradycardia and arrhythmia, bronchoconstriction, bronchosecretion		Headache, dizziness, drowsiness, anxiety	Mild	> 40
As above plus miotic pupils (unreactive to light), involuntary defecation and urination	Twitching of fine muscles, hyperreflexia, fasciculations	As above plus ataxia, psychosis, tremor, dysarthria, slurred speech	Moderate	20–40
	As above plus muscle weakness, reduced tendinous reflexes, paralysis affecting diaphragm and respiratory muscles	As above plus coma, convulsions, respiratory depression	Severe	< 20

rapidly than in erythrocytes where AChE activity is recovered via erythropoiesis. The level of activity of ChE should be carefully interpreted since the normal range in healthy subjects is relatively wide, rendering interpretation in individual patients difficult unless the results of previous estimations in the patient are available. Inhibition of ChE does not provide accurate information related to the clinical severity of the poisoning. Many OP insecticides, for example chlorpyrifos, demethon, and malathion, are apparently more potent inhibitors of ChE than they are of erythrocyte AChE and, consequently, ChE inhibition might occur to a greater extent than AChE inhibition (Jokanović, 2009).

The first 4–6 h are the most critical in acute poisoning with OP pesticides. If there is improvement in symptoms after initial treatment then the patient is very likely to survive if adequate treatment is continued (IPCS, 1998; Jokanović, 2009). The duration of effects is determined mainly by the properties of the compound: its liposolubility, the stability of the OP–AChE complex, and whether it is reactivatable after the use of cholinesterase reactivators such as pyridinium oximes.

The rate of spontaneous reactivation (Fig. 68.1, Reaction 2) can be accelerated by pyridinium oximes that have a chemical structure which "fits" the structure of the inhibited AChE. The oximes can only be of benefit as long as inhibited AChE is not completely converted to the aged form.

68.4 Antidotes in the treatment of organophosphorus poisoning

68.4.1 Atropine

Atropine acts by blocking the effects of excess concentrations of ACh at muscarinic cholinergic synapses following OP inhibition of AChE. Atropine is the initial drug of choice in acute OP poisoning that can relieve the following symptoms of OP poisoning: sweating, salivation, rhinorrhea, lacrimation, nausea, vomiting, and diarrhea. It can also help control bradycardia and circulatory depressions, dilating the bronchi, and abolish bronchorrhea. Atropine does not bind to nicotinic receptors and cannot relieve nicotinic effects of OPCs. In addition, there is evidence regarding the anticonvulsant properties of atropine in poisoning with soman and VX (McDonough et al., 1987; Zilker, 2005; Jokanović, 2009, 2015; Vučinić et al., 2018; Timperley et al., 2018).

The standard dosing of atropine depends on the severity of OP poisoning. The initial dose is usually 2 mg in an adult (0.02 mg/kg in a child) given every 5–10 min until hyperatropinization (flushing, dryness of the mouth, nose, lungs, and the skin, heart rate 80–100/min, normal blood pressure, mydriasis). The dose may be increased as required. Patients poisoned with OPs appear to be resistant to the toxic effects of atropine and may require relatively large doses of atropine administered over a prolonged period of time. According to IPCS (2002) in severe OP poisoning the total dose of atropine given during 5 weeks of treatment can be as high as 30,000 mg.

In an open-label randomized clinical trial, Abedin et al. (2012) compared the efficacy and safety of conventional bolus doses of atropine with individual incremental doses for atropinization followed by atropine infusion for management of OP poisoning. It was concluded that rapid incremental dose atropinization followed by atropine infusion reduced mortality and morbidity from OP poisoning and shortened the length of the total hospital stay and recovery. They recommended that incremental

atropine and infusion should become the treatment of choice for OP poisoning.

68.4.2 Diazepam

Benzodiazepines are CNS depressants, anxiolytics, and muscle relaxants. Their main site of action is at the gamma-aminobutyric acid (GABA) receptor. The $GABA_A$ receptor is a ligand-gated chloride ion channel and is part of a superfamily of receptors which also includes the nicotinic acetylcholine receptor and the glycine receptor. GABA is the major inhibitory neurotransmitter in the mammalian CNS. Benzodiazepines, including diazepam, alter GABA binding at the $GABA_A$ receptor site in an allosteric fashion but these drugs do not directly activate the receptors (Sellström, 1992; Marrs, 2004; Jokanović, 2015).

Currently, the most important anticonvulsant is diazepam. The combination of atropine and diazepam is more effective in reducing mortality than atropine or oxime alone. It was also shown that diazepam enhanced the efficacy of low doses of atropine. In the cholinergic nervous system, diazepam appears to decrease the synaptic release of ACh. The main consequence of the action of benzodiazepines in CNS is the hyperpolarization of neurons. This makes neurons significantly less susceptible to cholinergically induced depolarization. The ultimate result is cessation of the propagation of convulsions (Sellström, 1992; Marrs, 2004; Jokanović and Stepanović Petrović, 2016).

In patients poisoned with OPs, benzodiazepines may have a beneficial effect in reducing anxiety and restlessness, reducing muscle fasciculation, arresting seizures and convulsions, controlling apprehension and agitation, and possibly reducing morbidity and mortality when used in conjunction with atropine and an oxime. Diazepam should be given to patients poisoned with OPs whenever convulsions or pronounced muscle fasciculation are present. In severe poisoning, diazepam administration should be considered even before these complications develop. The recommended dose of diazepam in cases of OPS poisoning is 5−10 mg i.v. in the absence of convulsions and 10−20 mg i.v. in cases with convulsions, which may be repeated as required (Johnson and Vale, 1992; Jokanović, 2009, 2015).

It appears that other anticonvulsant benzodiazepines, such as midazolam, avizafone, and lorazepam, are just as effective in stopping nerve agent-induced seizures as diazepam in a hospital setting. In animal studies, midazolam was even more effective than diazepam (Bokonjić and Rosić, 1991; Timperley et al., 2018). The FDA and French army considered approval of midazolam as a treatment for nerve agent-induced seizures (Sidell et al., 2009; Masson, 2011; Jokanović, 2012, 2015). In addition to diazepam, Delacour and Dorandeu (2014) recommended administration of clonazepam in the management of severe OP poisoning.

Detailed therapeutic protocols used in the treatment of poisoning with OPs are presented in several excellent reviews (World Health Organization, 1986; Lotti, 1991; Bismuth et al., 1992; Johnson and Vale, 1992; Johnson et al., 2000; Marrs and Vale, 2006; Jokanović, 2009, 2012; Jokanović and Prostran, 2009; Jokanović et al., 2010; Balali-Mood and Saber, 2012; Vučinić et al., 2018; Timperley et al., 2018).

68.4.3 Oximes

Extensive studies over the past decades have investigated the mechanism of oxime action. There is convincing evidence that the antidotal potency of oximes is primarily attributed to their abilities to reactivate the phosphorylated cholinesterases. Oximes reactivate phosphorylated cholinesterases by displacing the phosphoryl moiety from the enzyme due to their high affinity for the enzyme and powerful nucleophilicity. The rate of reactivation depends on the structure of the phosphoryl moiety bound to the enzyme, the source of the enzyme, the structure and concentration of oxime that is present at the active site, the rate of postinhibitory dealkylation known as aging, and the steric hindrance effects between the oxime molecule and phosphoryl moiety attached to the active site of AChE (Jokanović and Stojiljković, 2006; Maxwell et al., 2008; Jokanović, 2015). Phosphorylated oximes are formed during the reactivation reaction and some of them appear to be potent inhibitors of AChE (Luo et al., 1999; Ashani et al., 2003; Worek et al., 2007). It was shown that phosphorylated oximes of 2-substituted pyridinium compounds (e.g., 2-PAM, HI-6) are unstable, while those of 4-pyridinium aldoximes are markedly stable (Ashani et al., 2003; Kiderlen et al., 2005; Worek et al., 2000) (Fig. 68.2).

Oximes bind to AChE as reversible inhibitors and form complexes with AChE either at the acylation (catalytic) site, at the allosteric site, or at both sites of the enzyme, and protect AChE from phosphorylation. When the reversible inhibitor binds to the catalytic site, the protection is due to direct competition between the OP and reversible inhibitor. Binding of a reversible inhibitor to the allosteric site induces indirect protection of the active site. Differences in the mechanisms of enzyme reactivation and protection demonstrate how stereochemical arrangements of oximes can play a role in the potency of their therapeutic efficacy. Direct pharmacological effects, such as direct reaction with OPs (Van Helden et al., 1996; Jokanović and Prostran, 2009; Jokanović, 2012), anticholinergic and sympathomimetic effects may also be relevant for the interpretation of antidotal potency of oximes.

Mono- and bispyridinium oximes are effective against OP-inhibited AChE in the PNS, but have limited

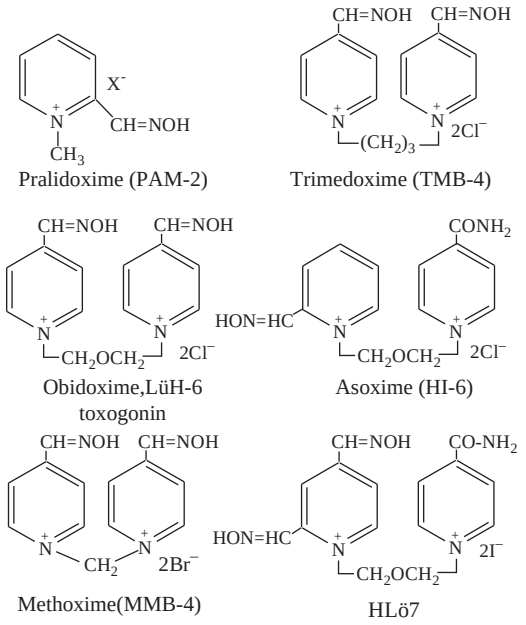

FIGURE 68.2 Chemical structure of pyridinium oximes used in treatment of OPC poisoning. X is an anion (in PAM-2 it can be Cl^-, I^-, or methanesulfonate).

penetration across the BBB. Limited penetration of HI-6 is due to its pharmacokinetic profile and the two quaternary nitrogen atoms in its structure. However, it appears that oxime penetration through the BBB is underestimated since soman can cause seizure-related opening of the BBB (Carpentier et al., 1990; Grange-Messent et al., 1999) and thus enable passage of higher oxime concentrations into the brain. Abdel-Rahman et al. (2002) have shown that 0.5−1.0 LD_{50} of sarin caused a dose-dependent increase in permeability of BBB in midbrain, brainstem, cerebrum, and cerebellum in rats 24 h after poisoning. Sakurada and coworkers (2003) have determined the amount of PAM-2 passing across the BBB at approximately 10% of the given dose, which may be effective in reactivation of OP-inhibited AChE in brain. Obidoxime concentrations in brain were estimated to be 3%−5% of plasma levels in rodents (Falb and Erdmann, 1969) and those of HI-6 to 10% of the serum highest concentration (Cassel et al., 1997). Several studies (Kassa; 2002; Shrot et al., 2009) suggested that such oxime concentrations in brain are apparently sufficient to produce biochemical and physiological effects in soman poisoning, particularly in specific brain regions, such as the medulla oblongata. These effects can contribute to survival following OP intoxication. Others claim that it is uncertain whether this amount is sufficient to reactivate nerve agent-inhibited AChE (Shih et al., 2011). When AChE reactivation was achieved in the pontomedullar region good therapeutic effects of oximes were observed and survival of poisoned animals correlated with the AChE activity in this region (Bajgar et al., 2007). In addition, it was suggested that in pralidoxime and obidoxime penetration through the BBB, a carrier-mediated transport mechanism might be involved (Lorke et al., 2008).

68.5 Pyridinium oximes in the management of poisoning with warfare nerve agents

68.5.1 Pralidoxime (PAM-2)

Pralidoxime was synthesized in the United States in 1955 (Wilson and Ginsburg, 1955). Its four salts, chloride (PAM-2 Cl), methiodide, methysulfate, and mesylate (P2S), were investigated and introduced into practice. PAM-2 is very effective in reactivating AChE inhibited with sarin or VX (Johnson and Stewart, 1970; Sidell and Groff, 1974; Harris and Stitcher, 1983; Mesić et al., 1991; Masuda et al., 1995; Nozaki and Aikawa, 1995), but was not successful in the reactivation of the tabun- or soman-inhibited enzymes (Inns and Leadbeater, 1983; Koplovitz and Stewart, 1994).

Because PAM-2, as a quaternary pyridinium salt, penetrates the BBB only to about 10% of its blood concentration, its prodrug pro-2-PAM (*N*-methyl-1,6-dihydropyridine-2-carbaldoxime hydrochloride), which is apparently rapidly oxidized in brain to PAM-2, was synthesized and tested in experimental animals. Pro-2-PAM provided some reactivation of sarin- and VX-inhibited AChE in the brain, blood, and peripheral tissues of guinea pig, which was reflected by a limited ability to block or terminate seizures elicited by these agents. Pro-2-PAM provided marginal reactivation of blood, heart, and spinal cord AChE inhibited by cyclosarin, but was not effective against cyclosarin-induced seizures (Shih et al., 2011). Pro-2-PAM was effective in the suppression and elimination of seizures/status epilepticus in DFP-treated guinea pigs. In addition, pro-2-PAM provided a significant reduction of neurological damage in DFP-poisoned guinea pigs. In both cases, PAM-2 was ineffective (DeMar et al., 2010). Clement (1979) reported that pro-2-PAM was effective in protecting mice poisoned with DFP and sarin, but very slightly effective against soman in guinea pigs. In summary, pro-2-PAM was not a significant improvement over PAM-2 with regard to prophylaxis against noted OPC. However, pro-2-PAM was less effective than PAM-2 in experimental poisoning with paraoxon in mice (Bošković et al., 1980; Jokanović and Stepanović Petrović, 2016).

When given together, atropine and PAM-2 act synergistically involving different mechanisms and the net effect is protection/treatment of animals poisoned with very high doses of certain OPs. Studies with nonhuman primates proved that the combination of atropine and

PAM-2 provided protection up to five times the LD_{50} of all known nerve agents except soman (Stojiljković et al., 2001) and against 128 LD_{50} of paraoxon (Murphy, 1986).

PAM-2 may be given by slow intravenous injection over 5−10 min, by intravenous infusion over 15−30 min, or by subcutaneous or intramuscular injection; it has also been given orally (Pralidoxime, 2009). The minimum therapeutic concentration of PAM-2 in plasma is 4 μg/mL and this level is reached in about 16 min after a single injection of 600 mg PAM-2 Cl (Pralidoxime, 2007).

In the treatment of OP poisoning, atropine and PAM-2 should be given as soon as possible and maintained as long as needed. When the effects of atropine become apparent, 1−2 g of PAM-2 chloride, iodide, or mesilate, should be given intramuscularly or intravenously and repeated after 1 h and then every 8−12 h if necessary. Alternatively, PAM-2 Cl may be given in an initial dose of 30 mg/kg by intravenous infusion over 20 min, or by intravenous injection over at least 5 min if pulmonary edema is present or infusion cannot be given; the initial dose is then followed by intravenous infusion at a rate of 8 mg/kg per hour (IPCS, 1998; Milatović and Jokanović, 2009; Pralidoxime, 2009). PAM-2 is relatively short acting (the apparent half-life of PAM-2 Cl is 74−77 min) and repeated doses may be needed, especially where there is any evidence of continuous absorption of the OPC (Pralidoxime, 2007). The treatment should be continued until no longer needed. This decision can be made on the basis of the clinical status of the patient, relatively high AChE activity in erythrocytes when compared to control values, and the absence of OPCs and/or OP metabolites in urine (Jokanović, 2009).

In some countries, autoinjectors are available for emergency use containing PAM-2, either alone or combined with atropine and/or avizafone, a water-soluble prodrug of diazepam (Pralidoxime, 2009). The French army is considering including water-soluble benzodiazepine midazolam in its autoinjector (Masson, 2011). Typical doses are 600 mg of PAM-2 Cl or 500 mg of PAM-2 mesilate given intramuscularly up to three times, depending on symptoms. In severe poisoning, PAM-2 may be given as a continuous infusion of 200−500 mg/h, titrated against response. A maximum dose of 12 g in 24 h has been suggested (Pralidoxime, 2009; Timperley et al., 2018).

PAM-2 has been very well tolerated in most cases (Pralidoxime, 2007). Adverse effects of PAM-2 in volunteers include dizziness, drowsiness, blurred vision, occasional diplopia, impaired accommodation, nausea, headache, tachycardia, hyperventilation, increased systolic and diastolic blood pressure, and muscular weakness. Tachycardia, laryngospasm, and muscle rigidity have been attributed to giving pralidoxime intravenously too quickly. Large doses of pralidoxime may cause transient neuromuscular blockade (Jager and Stagg, 1958; Sidell and Groff, 1971; Eyer, 2003; Pralidoxime, 2007, 2009). Forty to 60 min after intramuscular injection, mild to moderate pain may be experienced at the site of injection. Elevations in AST and/or ALT enzyme levels in blood were observed in one of six normal volunteers given 1200 mg of PAM-2 Cl intramuscularly, and in four of six normal volunteers given 1800 mg intramuscularly. Levels returned to normal in about 2 weeks (Pralidoxime, 2007). PAM-2 should be used cautiously in patients with renal impairment and a reduction in dosage may be necessary. Caution is also required in giving pralidoxime to patients with myasthenia gravis as it may precipitate a myasthenic crisis (Pralidoxime, 2009).

The clinical experience with the use of PAM-2 iodide, given with atropine and diazepam, in the treatment of the victims of Tokyo sarin attack in 1995 was very favorable (Sidell et al., 2009). However, PAM-2 should not be recommended as the drug of choice for the management of poisoning with all OPs due to its lack of efficacy against tabun and soman.

68.5.2 Trimedoxime (TMB-4)

TMB-4 Cl_2 was synthesized in the United States in 1957 (Poziomek et al., 1958). It is the only of the major bispyridinium oximes with a propylene bridge between the two pyridinium rings. It was shown that TMB-4 is a more potent reactivator of the DFP-inhibited AChE than PAM-2 (Hobbiger and Sadler, 1958) and better reactivator than LüH-6 in the case of the tabun-inhibited enzyme (Hobbiger and Vojvodić, 1966). TMB-4 was the first oxime that was efficient in the treatment of animals intoxicated with tabun (Schoene and Oldiges, 1973; Maksimović et al., 1980; Bokonjić et al., 1993). It could also protect animals poisoned with sarin or VX, but not those intoxicated with soman (Maksimović et al., 1980; Inns and Leadbeater, 1983; Binenfeld, 1986). While TMB-4 was the most toxic oxime among the "great four"—it was shown in mice that its LD_{50} is three, four, and eight times less than that for LüH-6, PAM-2 and HI-6, respectively (Clement, 1981).

68.5.3 Obidoxime (LüH-6, toxogonin)

After being introduced into medical practice in 1964, obidoxime showed significant potential as an antidote in poisonings with OPs (Erdmann and Engelhard, 1964). Given with atropine, obidoxime efficiently protected experimental animals against poisoning with tabun (Inns and Leadbeater, 1983; Maksimović et al., 1989), sarin (Inns and Leadbeater, 1983; Maksimović et al., 1989), and VX (Maksimović et al., 1989). Obidoxime was more effective than TMB-4 as an antidote against intoxication with tabun (Heilbronn and Tolagen, 1965). Similarly to PAM-2 and TMB-4, obidoxime was also inefficient in soman

poisoning in mice (Maksimović et al., 1980), guinea pigs (Inns and Leadbeater, 1983), and primates (Hamilton and Lundy, 1989). Obidoxime was more efficient than HI-6 against tabun intoxication. Similarly, obidoxime was more effective as a medical countermeasure following intoxication with most cholinesterase-inhibiting insecticides, while HI-6 was considered to be a better drug against soman-inhibited acetylcholinesterase (Aas, 2003). However, LüH6 was not a potent reactivator of tabun-inhibited human AChE (Worek et al., 2004; Lundy et al., 2011). In contrast to TMB-4, obidoxime, when administered with atropine in pyridostigmine-pretreated guinea pigs, could also provide some protection against soman (Inns and Leadbeater, 1983).

When administered to human volunteers by the intramuscular route, 5 mg/kg of LüH-6 produced a plasma concentration >4 mg/L, from 5 min after injection to 3 h (Sidell and Groff, 1970). Adverse effects of LüH-6 in male volunteers were described as pallor, nausea, burning sensation, headache, generalized weakness, sore throat, and paresthesia of the face (Simon and Pickering, 1976; Eyer, 2003; Marrs and Vale, 2006). Transient hepatotoxic effects of LüH-6 were reported in about 10% of severely poisoned patients (Marrs, 1991; Eyer, 2003).

Obidoxime can be given with atropine in the treatment of OP poisoning in a usual initial dose of 250 mg (4 mg/kg) by slow intravenous injection. This may be followed by intravenous infusion of 750 mg over 24 h, continued until the concentration of OP is below critical levels; alternatively, repeated doses of 4–8 mg/kg may be given at intervals of 2–4 h. It has also been given by intramuscular injection (Obidoxime chloride, 2009).

68.5.4 Asoxime (HI-6)

The first oxime that could reactivate soman-inhibited AChE and provide at least some protection to animals experimentally poisoned with this nerve agent was synthesized in 1966. It has been shown that HI-6 is more potent than LüH-6 and HS-6 [1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride] in the protection of various rodent species from intoxication with soman (Oldiges and Schoene, 1970; Inns and Leadbeater, 1983; Mesić et al., 1991; Lundy et al., 2011), as well as sarin and particularly VX (Maksimović et al., 1980; Inns and Leadbeater, 1983). HI-6 could not reactivate tabun-inhibited AChE (Clement, 1982; Ćetković, 1984; Kassa et al., 2008) and it was inefficient when used as the only oxime against poisoning with tabun (Maksimović et al., 1980; Inns and Leadbeater, 1983; Mesić et al., 1991; Bajgar, 2010). However, other studies found that HI-6 can provide a similar degree of protection against tabun and soman intoxication in primates (Hamilton and Lundy, 1989). It is also suggested that sufficiently high doses of HI-6 could even protect rats from multiple lethal doses of tabun (Lundy et al., 1989). HI-6 given with atropine protected guinea pigs from poisoning against 5 LD_{50} of soman or cyclosarin (Lundy et al., 2005). It was suggested that HI-6 is superior to the other oximes in treatment of cyclosarin-poisoned animals (Kassa, 2002).

The acute toxicity of HI-6 is the lowest among the six oximes (HI-6, PAM-2, TMB-4, LüH-6, HLö-7, MMB-4) (Maksimović et al., 1980; Clement, 1981; Rousseaux and Dua, 1989; Bartošova et al., 2006) with an LD_{50} value after i.m. administration to rats of 781 mg/kg.

HI-6 was introduced into clinical practice in Serbia in the early 1980s. It was administered to more than 200 patients poisoned with OP insecticides and volunteers. A clinical study with HI-6, administered intramuscularly at doses up to 500 mg to 22 healthy volunteers, revealed no adverse effects. However, with patients poisoned by several OP insecticides, HI-6 provided rapid reactivation of AChE in almost all cases except in dimethoate and phosphamidon poisoning (Kušić et al., 1985, 1991; Jovanović et al., 1990, 2010; Jokanović and Maksimović, 1995). Similar results regarding HI-6 tolerance were later found in a double-blind, placebo-controlled, single-dose study in 24 volunteers (Clement et al., 1995). Safety of HI-6 administration correlated with the recent report of Pohanka and his team (2011) who found that therapeutic doses of HI-6 are safe in dogs, but also indicating that 10-fold therapeutic doses can cause hyperglycemia.

Clinical studies have shown that HI-6 dosed at either 250 or 500 mg by the intramuscular route reached plasma concentrations >4 mg/L in 4–6 min. This concentration was maintained for 125 min following the lower dose of 250 mg and 200 min following the higher dose of 500 mg (Kušić et al., 1985; Jokanović and Maksimović, 1995).

HI-6 is considered to be a very promising bispyridinium oxime in medical treatment following exposure to most nerve agents. For these reasons HI-6 is involved in the equipment of the Czech, Slovak, Swedish, and Canadian armies as an antidote against nerve agent intoxication (Bajgar, 2010; Masson, 2011) and is under development in other countries (Lundy et al., 2011; Masson, 2011; Jokanović, 2012). A disadvantage of HI-6 compared to other available oximes is the lack of stability in aqueous solutions (Kušić et al., 1985; Aas, 2003).

In addition to the four pyridinium oximes that were introduced to clinical practice, the oximes HLö-7 and MMB-4 require attention after proving to be efficient against warfare nerve agents in animal studies.

68.5.5 HLö-7

Another important "Hagedorn oxime" (after LüH-6, HS-6, and HI-6), HLö-7 was also synthesized in Freiburg,

Germany, in 1986 (Löffler, 1986). Only HLö-7 could reactivate AChE inhibited by any of the four major nerve agents in vitro and in vivo (DeJong et al., 1989; Worek et al., 1994a,b, 1995), as well as AChE inhibited by cyclosarin (Lundy et al., 1992). In addition, HLö-7 more efficiently restored the neuromuscular transmission impaired by in vitro superfusion of the neuromuscular preparation with tabun, sarin, soman, or cyclosarin compared to PAM-2, LüH-6, and HI-6 (Alberts, 1990). It was found that HLö-7 induced a significant reactivation of AChE in mice diaphragm inhibited with tabun, sarin, soman, and cyclosarin (Clement et al., 1992). Both HI-6 and HLö-7 can antagonize sarin-induced hypothermia, proving that, when given with atropine, they can pass the BBB and gain access to the CNS (Clement, 1992; Clement et al., 1992), the toxicity of HLö-7 was 2.5 times higher than that of HI-6 (Clement et al., 1992). The cardiovascular tolerability of HLö-7 was similar, but still not as good as HI-6, at least in anesthetized guinea pigs (Worek and Szinicz, 1993). HLö-7 appears to be more effective than HI-6 against tabun and VX poisoning and less effective against sarin, soman, and cyclosarin intoxication (Lundy et al., 1992; Eyer et al., 1992). HLö-7-induced protection against tabun poisoning in guinea pigs was significantly better compared to HI-6, while HI-6 was only slightly more efficient than HLö-7 in soman poisoning (Melchers et al., 1994a,b). However, Kuča and coworkers (Kuča et al., 2005, 2011) reported that HLö-7 was not a good reactivator of tabun-inhibited AChE in vitro, possibly due to the presence of the lone electron pair on the tabun amide group.

The pharmacokinetic profile of HLö-7 was similar to that of HI-6. The mean absorption half-time of HLö-7 was about 14 min after intramuscular administration. Maximum HLö-7 concentration in plasma was reached after 30 min and the half-time of elimination was about 45 min (Eyer et al., 1992).

68.5.6 Methoxime (MMB-4)

MMB-4 is a structural analog of toxogonin, showing potent antimuscarinic activity (Amitai et al., 1980). It is a bispyridinium 4,4′-*bis*-aldoxime first described by Hobbiger and Sadler (Hobbiger and Sadler, 1959). MMB-4 is currently being proposed as a leading candidate for the replacement of PAM-2 as an oxime reactivator of nerve agent-inhibited AChE activity that can prevent lethality after warfare nerve agent exposure (Worek et al., 2010; Masson, 2011; Lundy et al., 2011). So far, the oxime MMB-4 has not undergone any clinical trials (Lundy et al., 2011).

MMB-4 was less toxic in mice (LD_{50} = 441 mg/kg, i.m.) than obidoxime (188 mg/kg, i.m.) but more toxic compared to HI-6 (671 mg/kg i.m.) (Bartošova et al., 2006). It was shown that MMB-4 was inferior to HI-6 in reactivation of peripheral AChE from soman-poisoned animals, although both oximes were better than PAM-2. In soman-poisoned rats MMB-4 was not successful in protection or reactivation of soman-inhibited AChE (Shih, 1993). Luo and coworkers (2008) found that MMB-4 was not a good reactivator of soman-inhibited AChE obtained from human and three monkey species where it was found to be less effective compared to HI-6. This is consistent with the findings reported in other studies (Lundy et al., 2011).

MMB-4 was also less effective in reactivation of cyclosarin-inhibited rat brain AChE and sarin-inhibited human or monkey AChE compared to HI-6 (Kuča and Patočka, 2004; Lundy et al., 2011). MMB-4 was found to be a weak reactivator of tabun-inhibited human AChE (Worek et al., 2004). However, experimental data indicate that MMB-4 was superior to PAM-2 in reactivating OP-inhibited AChE and in preventing lethality in OP-poisoned animals (Worek et al., 2010). It was also reported that MMB-4 was the most effective oxime in reactivation of ChE in blood and peripheral tissues in guinea pigs poisoned by sarin, cyclosarin, VX, and VR (Shih et al., 2010). In addition, MMB-4 was reported to be a better AChE reactivator than PAM-2 in paraoxon- and methylparaoxon-poisoned rats (Petroianu et al., 2006, 2007).

68.6 Pyridinium oximes in the management of poisoning with organophosphorus pesticides

The efficacy of pyridinium oximes has been demonstrated in experimental conditions and in many cases of patients poisoned with OP insecticides who have been treated in European clinics. In those cases the recommendations for PAM-2 dosing proposed by the WHO were followed (IPCS, 1998). Contrary to these findings, reports from Asia indicate that PAM-2 treatment was not sufficiently effective in their patients. However, in their studies PAM-2 was not used as recommended by the WHO (Cherian et al., 1997, 2005; De Silva et al., 1992; Johnson et al., 1996; Singh et al., 1995). In addition, these studies were apparently poorly designed due to suboptimal dose, short duration of treatment, long delay between patient exposure and PAM-2 administration, and the chemical structure of OP pesticides not being taken in account (Jokanović, 2009; Jokanović et al., 2010).

A particular problem in interpreting the beneficial role and efficacy of pyridinium oximes in clinical practice is a relative lack of published data, especially those evaluated in controlled clinical trials. Studies related to the efficacy of oximes in clinical settings showed the heterogeneity of

therapeutic approaches (i.e., dose regimen, oxime choice, and final outcome of the treatment) (Jokanović, 2009).

Jokanović et al. (2010) reviewed the experience gained in clinical management of 296 patients poisoned with OP insecticides at the Clinic of Toxicology of the National Poison Control Center in Military Medical Academy in Belgrade during the period of 1998–2007. Poisoning with dimethyl OP was confirmed in 246 patients (malathion, 153; dimethoate, 69; dichlorvos, 4; fenitrothion, 6; monocrotophos, 8), with diethyl OP in 38 patients (diazinon, 21; parathion, 9; phorate, 1; phoxim, 1; phosalone, 3; chlorpyriphos, 2; quinalphos, 1) and in 12 patients in whom OP was not fully identified. The majority of poisonings (92%) was due to deliberate ingestion of OP insecticides. Accidental inhalational poisonings were registered in 3.8% patients, accidental ingestion in 4.1% patients, and there were two cases of deliberate intramuscular and intravenous application of OP. Suicidal poisonings were usually oral, and the severity of poisonings depended on the toxicity, the amount of OP insecticides taken, and the time from exposure to admission to the Clinic of Toxicology and initiation of treatment. In this study 15.9% of OP-poisoned patients died, which was consistent with the findings of other authors (Eddleston et al., 2008), who reported case fatality generally greater than 15%. About 42% of the deceased patients died within 7 days, and lethal outcome occurred within hours in 18% patients due to the severity of poisoning.

In this study, 74 patients were treated with pralidoxime methylsulfate (Contrathion). The dosage regimen for severely poisoned patients included a loading dose of 400 mg by slow intravenous infusion, subsequently 200 mg/h continuous intravenous infusion administered in the first day, and continued as long as clinical signs of poisoning and OP insecticide and/or its metabolite were present in urine and blood. In moderate poisoning, 400 mg of Contrathion was followed by 400 mg/3–4 h. However, due to the economic crisis in Serbia, the Clinic of Toxicology did not have oxime available for several years and the patients were treated with atropine and diazepam only. In patients treated with atropine and oxime or atropine only, there were no differences in mortality. Atropine consumption was lower in the group of patients treated with an oxime, but the difference was significant only in the group of severely poisoned patients, where it was reduced by 30%. An update of the study was recently reported by Vučinić et al. (2018).

Eddleston et al. (2005) conducted a prospective study on 802 patients self-poisoned with chlorpyrifos, dimethoate, or fenthion. Compared with chlorpyrifos (8.0%), the proportion of fatalities was significantly higher with dimethoate (23.1%) or fenthion (16.2%) than was the proportion requiring endotracheal intubation (chlorpyrifos 15.0%, dimethoate 35.2%, fenthion 31.3%). Patients poisoned by the diethyl OP pesticide (chlorpyrifos) responded well to pralidoxime, whereas those to the two dimethyl OP pesticides (dimethoate, fenthion) responded poorly. The poor efficacy of PAM-2 in the treatment of human dimethoate and fenthion poisonings was in agreement with experimental studies reported by Jokanović and Maksimović (1995) who found that the antidotal efficacy of obidoxime, trimedoxime, PAM-2, and HI-6, when given with atropine and diazepam in rats dosed with 2 LD_{50} of the dimethoate was low. However, there was a discrepancy between fenthion-poisoned patients and animals in that PAM-2 was ineffective as an antidote in patients, whereas the four oximes showed considerable efficacy in rats.

In a randomized controlled trial, Pawar et al. (2006) studied the effects of very high doses of PAM-2 iodide (2 g loading dose, then 1 g either every hour or every 4 h for 48 h, then 1 g every 4 h until recovery) in 200 patients with moderate OP poisoning (excluding severely ill patients). The OP pesticides involved were chlorpyrifos (diethyl OP) and dimethoate (dimethyl OP). The dosing regimen was associated with reduced case fatality, fewer cases of pneumonia, and reduced time on mechanical ventilation. This study suggests that large doses of PAM-2 could have a positive effect on patients if they are treated early and have good supportive care.

An interesting clinical trial where PAM-2 chloride was investigated in 235 patients self-poisoned with OP insecticides was reported by Eddleston and coworkers (2009). The patients were randomized to receive PAM-2 ($n = 121$) or saline placebo ($n = 114$). PAM-2 produced substantial and moderate erythrocyte AChE reactivation in patients poisoned by diethyl and dimethyl compounds, respectively. Mortality was insignificantly higher in patients receiving PAM-2: 30/121 (24.8%) receiving PAM-2 died, compared with 18/114 (15.8%) receiving placebo. The authors concluded that, despite clear reactivation of erythrocyte AChE in diethyl OP insecticide-poisoned patients, they found no evidence that treatment with PAM-2 improves survival or reduces the need for intubation in patients with OP insecticide poisoning. However, this study suffers from a major methodological problem of randomization as the authors wrote that "more severely poisoned patients were allocated to pralidoxime" than to the placebo treatment. Based on this statement it is expected that more patients would die in the group treated with PAM-2 despite the oxime treatment. This was supported by another sentence present in this report that "patients died sooner in the pralidoxime group." In addition, there were other problems with this study such as the lack of atropine inclusion in both groups. The World Health Organization (1986) recommends treatment of OP-poisoned patients with atropine, PAM-2, and diazepam, and not only with PAM-2. There were also a relatively

small number of patients included in the study, relatively long and variable period between self-poisoning and administration of PAM-2 (mean 4.3 h, intervals 2.9−7.8 h) probably having an impact on aging of phosphorylated AChE, ethical issues such as why the patients in placebo group were allowed to die without the best possible treatment according to the WHO recommendations. Because of these and other problems, the results of this study shall be considered with caution.

Kušić et al. (1991) have tested the oxime HI-6 in OP pesticide poisoning in 60 patients. HI-6 was administered four times a day as a single 500 mg i.m. injection with atropine and diazepam treatment. Oxime therapy was started on admission and continued for 2−7 days. Most patients were treated with HI-6 and nine severely poisoned patients with quinalphos were treated with PAM-2 chloride (1000 mg four times per day). HI-6 rapidly reactivated human erythrocyte AChE inhibited by diethoxy OPs (phorate, pyridaphenthion, quinalphos) as well as that inhibited by dichlorvos, a dimethoxy OP. AChE inhibited with other dimethoxy OPs, dimethoate and phosphamidon, was reported to be resistant to HI-6 treatment, whereas reactivation with malathion was slow, with a reactivation half-time of 10 h. Both HI-6 and PAM-2 successfully reactivated AChE in quinalphos-poisoned patients, with HI-6 acting as a faster AChE reactivator than PAM-2.

Willems et al. (1993) reported that ethylparathion and methylparathion poisoning could be effectively treated with PAM-2 methylsulfate (plasma concentrations 4 mg/L) and atropine when pesticide concentrations in plasma were relatively low. In severe poisoning with pesticide levels in plasma above 30 μg/L, high PAM-2 concentrations in plasma (14.6 mg/L) did not provide any improvement. In addition, PAM-2 at concentrations of 6.3 mg/L was not effective in AChE reactivation in dimethoate poisoning where AChE was inhibited with its active metabolite omethoate.

It was reported that in parathion poisoning, an obidoxime dose of 250 mg i.v. administered as a bolus followed by infusion of 750 mg/day was effective, but AChE reactivation following severe poisoning did not occur until the concentration of paraoxon in plasma became low (Thiermann et al., 1997, 1999). Oxydemeton methyl poisoning responded to obidoxime therapy only when the oxime was instituted shortly after poisoning. In cases when obidoxime treatment was started too late, there was no reactivation of erythrocyte AChE and one of six patients died.

Nine patients intoxicated with OP pesticides were treated with PAM-2 methylsulfate (Contrathion) using a dose of 4.42 mg/kg as a bolus injection followed by continuous infusion of 1.14 mg/kg/h. In patients poisoned with ethylparathion or methylparathion, AChE reactivation could be obtained at low oxime concentrations (2.88 mg/L). In others, however, an oxime concentration as high as 14.6 mg/L was ineffective. The therapeutic effect of the oxime apparently depended on the plasma concentrations of the OP pesticides. Due to AChE reinhibition, reactivation was absent as long as these concentrations remained above 30 μg/L (Aragao et al., 1996).

In a clinical study involving 63 patients poisoned with organophosphorus insecticides, patients were divided into three groups: one was treated with atropine only, while the other two received atropine and either PAM-2 or obidoxime. Initial and maintenance intravenous doses for PAM-2 were 30 mg/kg and 8 mg/kg/h, respectively, and 8 mg/kg and 2 mg/kg/h, respectively, for obidoxime. The major clinical findings or AChE activities at the time of admission did not show statistically significant differences among the groups. Although the severity of intoxications, based on respiratory complications and duration of hospitalization, was higher in the atropine plus oxime groups, 12% and 50% of patients in the atropine and atropine plus obidoxime groups died, respectively. No mortality was found in the PAM-2 plus atropine group. The incidence of recurrent twitching and convulsions, repeated respiratory arrest, required mechanical respiration, required intensive care unit therapy, and duration of hospitalization were lower in the atropine plus obidoxime group than in the atropine plus PAM-2 group. Three of the patients who received the obidoxime combination therapy developed hepatitis and two of these died due to hepatic failure, which may indicate overdosage of obidoxime (Balali-Mood and Shariat, 1998).

In the study reported by Tsai and coworkers (2007) PAM-2 was administered to 56 patients within 48 h of OP pesticides poisoning. The average duration of PAM-2 treatment was 5.5 ± 4.9 days and the mean total dose was 16.4 ± 12.4 g. The mortality and length of stay did not improve with PAM-2 treatment. Days spent on ventilator support were decreased in patients treated with PAM-2 (6.7 ± 1.9 days) compared to those who were not (23.0 ± 4.8 days). However, there are many limitations in this study. For example, there was a relatively small number of treated patients, and a lack of data regarding the OP pesticides involved, dose taken, and AChE inhibition.

AChE inhibited by several OP pesticides, including dimethoate, demethon, triamiphos, ethoprophos, profenofos, fenamiphos, and pyridafenthion, was resistant to any attempt at reactivation with any oxime, probably due to the variations in phosphoryl moiety and distribution of electronic charge (Bismuth et al., 1992; Jokanović and Maksimović, 1995; Jokanović, 2009, 2012; Jokanović and Stepanović Petrović, 2016).

Jokanović and Maksimović (1995) studied the acute oral toxicity of 25 OP insecticides and one OP fungicide (pyrazophos) in the rat and the efficacy of antidotal

treatment involving TMB-4, LüH-6, PAM-2, and HI-6 (given with atropine and diazepam 1 min after poisoning) in animals dosed with 2 LD_{50} of the OP insecticides. The success of therapy was dependent on the chemical structure of the OPs. The oximes were potent antidotes in poisoning with the insecticides having phosphate structure, and provided some extent of antidotal protection in poisoning with phosphonates, phosphorothiolates, phosphorothionates, and phosphorodithioates. However, none were effective antidotes against dimethoate and pyridafenthion. This study has shown that TMB-4 was the most effective pyridinium oxime in the treatment of OP insecticide poisoning.

68.7 Concluding remarks and future directions

Despite the enormous efforts devoted to the synthesis and development of new pyridinium oximes as potential antidotes against poisoning with OPs in the past several decades, there have been no major advances in designing compounds that would be effective against nerve agents and other OPs. Only four compounds so far are applicable in human medicine. However, they differ in their activity in poisoning with warfare nerve agents and OP insecticides. There is still no universal broad-spectrum oxime capable of protecting against all known organophosphorus compounds.

HI-6 seems to be a promising choice for nerve agent poisoning because it was able to protect experimental animals from toxic effects and improve the survival of animals poisoned with sublethal doses, especially in the cases of soman and cyclosarin. HI-6 was also effective in poisoning with OP insecticides. MMB-4 is currently being proposed as a leading candidate for replacement of PAM-2 as an oxime reactivator of nerve agent-inhibited AChE activity that can prevent lethality after warfare nerve agent exposure. Available experimental evidence suggests that there are no clinically important differences between pralidoxime, obidoxime, and HI-6 in the treatment of nerve agent poisoning. On the other hand, obidoxime appears to be a good choice for the treatment of poisoning with OP insecticides. However, it is necessary to rigorously test these statements in properly designed randomized clinical trials.

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Chapter 69

Novel cholinesterase reactivators

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69.1 Introduction

The continuous threat of organophosphorus (OP) nerve agent misuse supports the development of antidotes with enhanced potency. In this chapter, we focus on antidotal therapy with cholinesterase reactivators. Although this group of compounds has been known for more than a half-century, there is no single compound that might counteract every nerve agent's lethal effects. This chapter describes in detail the design and synthesis of the cholinesterase reactivators that have been developed within the last decade. Within the design of novel molecules, special emphasis is placed on the structural requirements (structure−activity relationship) of these compounds. Finally, several new trends in the development of new reactivators are discussed.

69.2 OP AChE inhibitors

OP compounds are widely used in agriculture as pesticides (e.g., dichlorvos, diazinon, chlorpyrifos, and parathion), in industry and technology as softening agents and lubricant additives, and in the medical and veterinary fields as therapeutic agents (Gupta, 2006, 2015). Some OPs are declared as chemical warfare agents (CWAs), nerve agents, or both. Sarin, cyclosarin, soman, tabun, *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX), and Russian VX are well-known members of the OP nerve agent family (Table 69.1; Marrs, 1993; Bajgar, 2004, Gupta, 2015).

The history of nerve agents began prior to World War II in Germany. The first-known nerve agent, tabun (*O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate), was synthesized in the laboratories of IG Farben in Germany by Dr. Gerhard Schrader in 1936. Although the original aim of Schrader's studies was synthesizing pesticides, German authorities identified the deadly potential of OPs, and subsequently, many nerve agents, such as sarin, cyclosarin, and soman, were developed. A few decades later, the nerve agent VX was developed in the United Kingdom. Further developed nerve agents include intermediate volatile agents, an example of which is Agent GP, and A-agents or their precursors, so-called Novichoks (Fig. 69.1; Halamek et al., 2007; Nepovimova and Kuca, 2018).

During the Cold War, nerve agents were stored and prepared for potential military use, but were not used in military conflicts. However, they were misused by Saddam Hussein in Iraq in the Kurdish village of Birjinni (1988) and by the Japanese Aum Shinrikyo sect in Matsumoto (1994) and Tokyo (1995) (Tu, 2000). More recently, sarin was several times misused within the Syrian conflict (2013−18; John et al., 2018) and an agent from the Novichok family was proposed to be the cause of the intoxications of Sergei and Yulia Skripal in the UK (2018) (Nepovimova and Kuca, 2018).

Nerve agents phosphonylate serine Ser203 at the esteratic part of the active site of enzyme AChE (EC 3.1.1.7). AChE plays a key role in termination of the action of a neurotransmitter acetylcholine (ACh) in the peripheral nervous system and central nervous system, and persistent inhibition of AChE can lead to life-threatening consequences (Marrs, 1993).

Depending on the particular nerve agent, AChE is further irreversibly phosphylated via a time-dependent process called *aging*, defined as dealkylation of the phosphyl adduct to give a negatively charged adduct that is stabilized by interaction with catalytic His440 (Millard et al., 1999; Carletti et al., 2010). Aged enzymes cannot be restored by any nucleophilic reactivators. Because of the irreversible inhibition of AChE, the enzyme is not able to fulfill its physiological role in the organism—namely, splitting the neuromediator ACh at the synaptic clefts. Subsequently, ACh accumulates at the cholinergic synaptic junctions and persistently stimulates cholinergic receptors (Marrs, 1993).

The acute toxicity of nerve agents is usually attributed to excessive cholinergic stimulation caused by excess accumulation of ACh, followed by subsequent overstimulation

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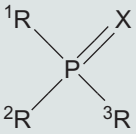
TABLE 69.1 Selected AChE inhibiting OP nerve agents, pesticides, and prototype compounds.



Compound	^{1}R	^{2}R	^{3}R	X	Class
Tabun	*O*-Et	N-Me_2	CN	O	NA
Sarin	*O*-isopropyl	Me	F	O	NA
Soman	*O*-(3,3-dimethylbut-2-yl)	Me	F	O	NA
Cyclosarin	*O*-cyclohexyl	Me	F	O	NA
VX	*S*-[(2-diisopropyl)aminoethyl]	Me	*O*-Et	O	NA
Russian VX	*S*-[(2-diethyl)aminoethyl]	Me	*O*-isobutyl		NA
DFP	*O*-isopropyl	*O*-isopropyl	F	O	Pesticide
DDVP	*O*-(2,2-dichloroethenyl)	*O*-Me	*O*-Me	O	Pesticide
Paraoxon	*O*-(4-nitrophenyl)	*O*-Et	*O*-Et	O	Pesticide
Parathion	*O*-(4-nitrophenyl)	*O*-Et	*O*-Et	S	Pesticide

Et, ethyl; Me, methyl.

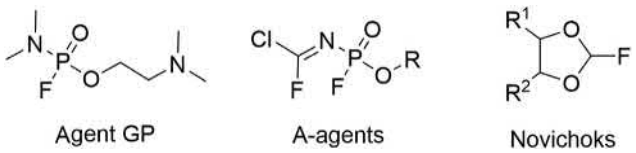


FIGURE 69.1 Proposed structure of new nerve agents (Halamek et al., 2007, Nepovimova and Kuca, 2018).

of cholinergic pathways and desensitization of cholinergic peripheral and central receptor sites (Bajgar, 2004). Symptoms of intoxication are as follows: when an individual is exposed to low amounts of a nerve agent, the initial symptoms include runny nose, contraction of the pupils, miosis, deterioration of visual accommodation, headache, slurred speech, nausea, hallucination, pronounced chest pain, and an increase in the production of saliva (muscarinic central and peripheral symptoms). At higher doses, the aforementioned symptoms are more pronounced, and coughing and breathing problems also occur. The individual may then begin to have convulsions (nicotinic symptoms) and may experience impaired ventilation, coma, and death. At even higher doses, an exposed individual would almost immediately go into convulsions and die from respiratory and cardiovascular failure. The initial stages of symptoms of an individual exposed to a nerve agent may vary depending on the particular nerve agent and the amount of agent the individual was exposed to (Bajgar, 2004).

69.3 Acetylcholinesterase (AChE; EC 3.1.1.7)

AChE is a serine hydrolase enzyme that belongs to the esterase family within higher eukaryotes. Enzymes of this family act on different types of carboxylic esters. The biological role of AChE is termination of transmission impulses at cholinergic synapses within the nervous system by rapid hydrolysis of neurotransmitter ACh. The monomer of AChE, with a molecular weight around 60,000, is an ellipsoidal molecule whose size is approximately $45 \times 60 \times 65$ angstrom (Å), consisting of a 12-strand, central, mixed β-sheet surrounded by 14 α-helices (Sussman et al., 1991). Each monomer contains one catalytic center composed of two compartments: the esteratic subsite containing the catalytic triad and the anionic subsite that accommodates the positive quaternary compartment of ACh. In human AChE, the esteratic subsite contains the catalytic machinery of the enzyme: a catalytic triad of Ser203, His447, and Glu334. The anionic subsite is defined by Trp86, Tyr337, and Phe338. Its role is to orient the charged part of ACh that enters the active center. This role is the main function of Trp and Tyr residues (Bourne et al., 2003). The recent rendition of the X-ray structure for AChE places the active catalytic site deep within a gorge-like fold of the protein. The aromatic gorge in the protein is approximately 20 Å deep and penetrates halfway into the enzyme. The active site lies at the

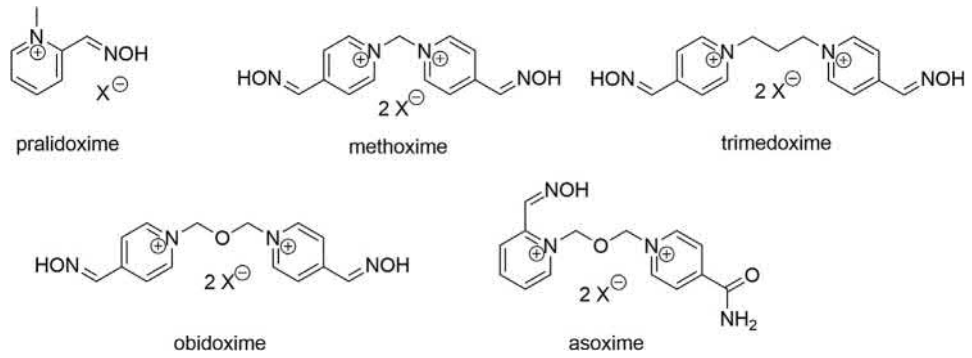


FIGURE 69.2 Standard AChE reactivators (Musilek et al., 2011).

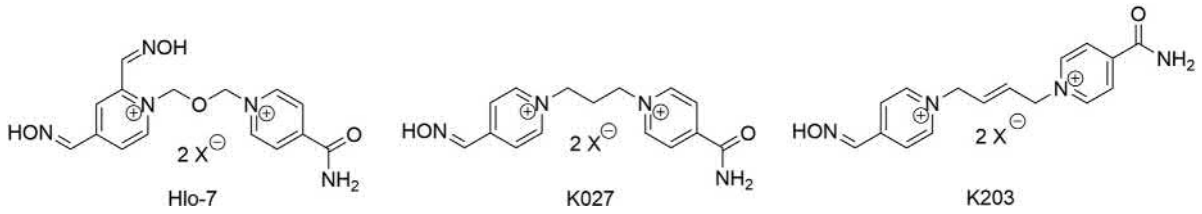


FIGURE 69.3 Selected AChE reactivators that are under development.

base of this gorge only 4 Å above the base, leading some to label this the "active gorge". The aromatic gorge is a more appropriate term because 40% of its content is composed of 14 aromatic residues, which is highly conserved from different species of AChE (Harel et al., 1993). The high aromatic content may explain studies that have proposed hydrophobic and anionic binding sites independently on the active site. Only a few acidic residues are present within the gorge. The aromatic residues play an important role in stabilization of the enzyme–substrate complex. Both electrostatic and hydrophobic effects are important here. The electrostatic potential map of AChE suggests that this enzyme, like the other enzymes with charged substrates, steers its substrate toward its gorge and into the active site.

The second anionic site of AChE, the so-called peripheral anionic site, is located at the active center gorge entry and encompasses overlapping binding sites for different activators and inhibitors. The peripheral anionic site consists of residues Tyr72, Trp286, and Tyr341. Binding of ligands to these residues may be key to the allosteric modulation of AChE catalytic activity (Bourne et al., 2003).

69.4 Antidotes for AChE inhibited by OP compounds

Antidotes developed for treatment of nerve agent intoxication can be divided into two types: prophylaxis, as preexposure administration of antidotes; and postexposure treatment, consisting of anticholinergic drugs, AChE reactivators, and anticonvulsants. The best-known prophylactic means are carbamates (e.g., pyridostigmine and physostigmine), oximes (e.g., asoxime and the Transant patch), and bioscavengers (e.g., butyrylcholinesterase (BChE), paraoxonase, and phosphotriesterase). The main drugs used for postexposure treatment are anticholinergic agents (functional antidotes) that antagonize the effects of accumulated ACh at cholinergic synapses, and AChE reactivators (called *oximes* concerning their functional oxime group) which restore AChE inhibited by the OP inhibitor (causal antidotes). Their effects are synergistic. Centrally active drugs such as benzodiazepines (e.g., diazepam or avizafone) are also used as anticonvulsants (Bajgar, 2004).

Pralidoxime chloride or mesylate (2-PAM), obidoxime chloride (LüH-6), methoxime chloride or mesylate (MMC-4 or MMB-4), trimedoxime chloride (TMB-4 or TMC-4), and asoxime chloride or mesylate (HI-6) are considered the most important commercially available AChE reactivators (Fig. 69.2). Other AChE reactivators are currently under development in different countries, including HLö-7, K027, and K203 (Fig. 69.3; Gorecki et al., 2019).

From a chemical point of view, standard AChE reactivators are monoquaternary or bisquaternary pyridinium salts bearing a functional oxime group in their molecules. The oxime in the form of oximate is able to split the bond between the OP and enzyme. Consequently, free-functioning AChE is released. Unfortunately, none of five commercial AChE reactivators is effective against all known nerve agents and OP pesticides (Table 69.2; Kuca et al., 2007). However, there are some novel experimental reactivators that might bring additional benefit to the postexposure antidotal treatment.

TABLE 69.2 Reactivation potencies of five commercially available oximes (Kuca et al., 2007).

	Pralidoxime	Trimedoxime	Obidoxime	Methoxime	Asoxime
VX	34/ 0	66/ 10	79/ 8	59/ 0	28/ 13
	++ / −	++ +/+	++ +/+	++ +/−	++/+
Russian VX	70/ 0	30/ 4	66/ 17	79/ 36	42/ 53
	++ +/−	++/−	++ +/+	++ +/++	++ +/++ +
Sarin	31/ 0	54/ 7	26/ 3	43/ 0	47/ 50
	++/−	++ +/+	++/−	++ +/−	++ +/++ +
Cyclosarin	4/ 0	0/ 0	4/ 2	37/ 90	70/ 71
	−/−	−/−	−/−	++/++ +	++ +/++ +
Tabun	4/ 0	28/ 10	37/ 28	0/ 0	2/ 4
	−/−	++/+	++/++	−/−	−/−
Soman	0/ 0	0/ 0	4/ 1	24/ 4	5/ 3
	−/−	−/−	−/−	++/−	+/−
Chlorpyrifos	38/ 4	66/ 38	63/ 35	45/ 10	20/ 11
	++/−	++ +/++	++ +/++	++ +/+	++/+

Reactivation (%) at oxime concentration 10^{-3} M/10^{-5} M.

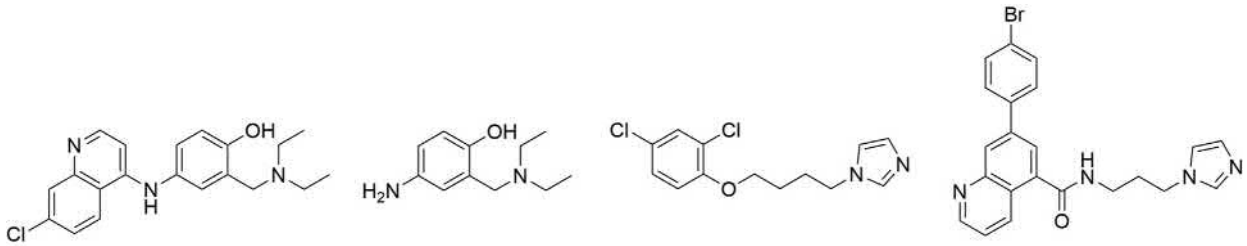


FIGURE 69.4 Uncharged nonoximes (Katz et al., 2015).

69.5 Design and synthesis of new AChE and BChE reactivators

To date, there are many scientific institutions around the world that are interested in the design, synthesis, and improvement of new antidotes against CWAs, especially nerve agents. This is because of the continuous threat of nerve agent misuse by terrorists. Finding a suitable oxime that is sufficiently effective against OP-inhibited AChE or >BChE (regardless of the type of OP compound) is a very important task. There are several reviews dealing with the synthesis of new cholinesterase reactivators (e.g., Musilek et al., 2007, 2011; Mercey et al., 2012; Korabecny et al., 2014; Sharma et al., 2015; Gorecki et al., 2016, 2017; Malinak et al., 2018a; Franjesevic et al., 2019). Several studies related to the design and synthesis of novel reactivators since 2013 are further discussed. The prepared compounds were sorted according to their chemical structure to uncharged non-oxime reactivators, uncharged oxime reactivators, and mono- or double-charged oximes reactivators.

69.6 Uncharged non-oxime reactivators

Katz et al. (2015) discovered Mannich phenol bases without oxime moiety (**1**−**4**; Fig. 69.4) that were studied on a reactivation mechanism and tested on NIMP-, SIMP-, paraoxon-, and DFP-inhibited human, mouse, and guinea pig AChE. Compound ADOC (**2**) was found to have a reactivation effect better than pralidoxime and further compound SP134 (**3**) was selected due to better reactivation of paraoxon and DFP, whereas compound SP138 (**4**)

was chosen due to better reactivation of NIMP and SIMP surrogates.

Cadieux et al. (2016) explored a series of compounds missing oxime moiety derived from ADOC (**5**–**6**; Fig. 69.5) and evaluate them on sarin-, cyclosarin-, VX-, and VR-inhibited hAChE. The phenolic fragment was found to be important for reactivation ability, however ADOC (**2**) resulted as the best compound and only worse or comparable to pralidoxime reactivation ability.

Katz et al. (2018) reported screening for compounds without oxime moiety (**7**–**8**; Fig. 69.6) that were tested on mouse AChE or human BChE inhibited by paraoxon. The non-oxime compounds were found to be weak reactivators of phosphylated AChE or BChE comparable with pralidoxime in some cases.

de Koning et al. (2018) developed compounds without oxime moiety derived from ADOC (**9**; Fig. 69.6) that were assayed on hAChE inhibited by VX, sarin, cyclosarin, tabun, and paraoxon. Some compounds showed similar reactivation ability to parent ADOC (**2**) molecule, but they were unfortunately not compared to a standard oxime reactivator. The inhibitory ability of novel compounds varied from low to high μM range. This study was further expanded by Horn et al. (2018) and four compounds including ADOC (**9**) were tested on hAChE inhibited by VX, sarin, cyclosarin, and paraoxon. One compound showed some ability to reactivate all OP species, but in most cases the effect was much lower than asoxime.

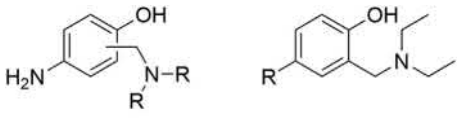


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FIGURE 69.5 ADOC analogues (Cadieux et al., 2016).

69.7 Uncharged oxime reactivators

A series of seven novel uncharged compounds (Renou et al., 2013) combining 3-hydroxy-2-pyridinealdoxime with different peripheral site ligands was prepared and examined for VX- and tabun-inhibited human AChE (**10**; Fig. 69.7). Concerning in vitro reactivation of VX-hAChE, they were more efficient than pralidoxime, but less efficient than obidoxime and asoxime. Regarding tabun-inhibited AChE, only one compound reached a reactivation potency comparable to pralidoxime.

Kovarik et al. (2013) published a huge series of uncharged hydroxyiminoacetamides and hydroxyiminoimidazoles (**11**–**13**; Fig. 69.7) that were referred to as centrally acting oximes for OP-inhibited AChE. Among 135 novel oximes, compound RS194B (**4**) was highlighted to efficiently reactivate sarin, cyclosarin, and VX in a rate similar to pralidoxime under in vitro conditions.

Sit et al. (2014) developed uncharged or monocharged 2-hydroxyiminoimidazoles (**14**; Fig. 69.8) that were tested on BChE inhibited by analogs of sarin, cyclosarin, VX, and paraoxon in order to prepare an effective pseudocatalytic bioscavenger. The reactivation of hAChE was found to be slow, but several compounds were found to be better than TAB2OH (**38**) or commercial standard pralidoxime for reactivation of hBChE inhibited by cyclosarin and VX.

Renou et al. (2014) designed uncharged tryptoline-3-hydroxypyridinaldoximes (**15**; Fig. 69.8) to reactivate human acetylcholinesterase inhibited by VX, tabun, and paraoxon or human butyrylcholinesterase inhibited by VX. Two compounds were highlighted for reactivation of VX-, tabun-, and paraoxon-inhibited AChE in all cases better than pralidoxime and similar or slightly better than commercial standards trimedoxime, obidoxime, or asoxime for specific OPs. The reactivation of VX-inhibited BChE was found to be far better than pralidoxime, obidoxime and asoxime.

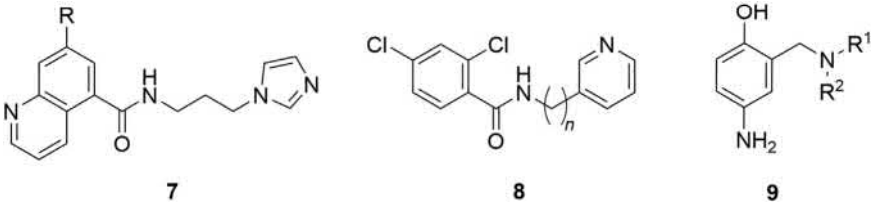

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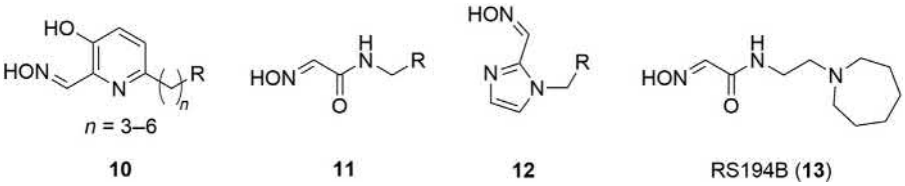
FIGURE 69.6 Uncharged nonoximes and ADOC analogs (Katz et al., 2018; de Koning et al., 2018).



10 11 12 RS194B (13)

FIGURE 69.7 Uncharged 3-hydroxy-2-pyridinealdoximes and hydroxyiminoacetamides (Renou et al., 2013; Kovarik et al., 2013).

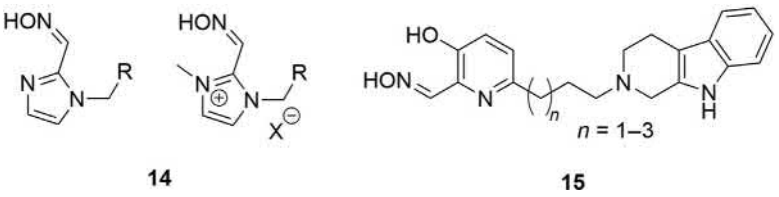


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FIGURE 69.8 2-hydroxyiminoimidazoles and tryptoline-3-hydroxypyridinaldoximes (Sit et al., 2014; Renou et al., 2014).

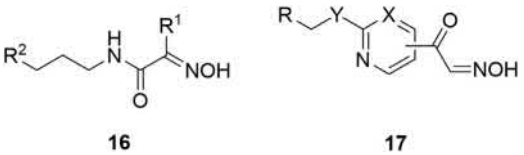

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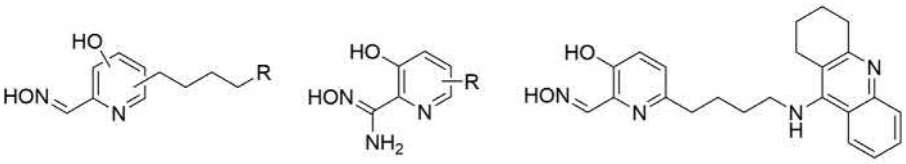
FIGURE 69.9 Amide oximes and ketoximes (McHardy et al., 2014).



18 19 20

FIGURE 69.10 Tetrahydroacridine pyridine-aldoximes/amidoximes (Kliachyna et al., 2014).

McHardy et al. (2014) developed uncharged amide oximes and ketoximes (**16**–**17**; Fig. 69.9) that were designed to reactivate cyclosarin-inhibited AChE. The reactivation results were only compared to monoisonitrosoacetone (MINA) that does not belong among the standard oximes used for OP poisoning treatment. Some novel reactivators were found to be potent AChE inhibitors in the μM or nM range.

Kliachyna et al. (2014) presented uncharged tetrahydroacridine pyridine-aldoxime and -amidoxime hybrids (**18**–**20**; Fig. 69.10) and tested them for reactivation of AChE inhibited by VX, tabun, and paraoxon. One compound (**20**) resulted as a powerful reactivator of all tested OPs under in vitro conditions when compared to pralidoxime, trimedoxime, obidoxime, or asoxime. Some novel reactivators, including the most potent compound (**20**), were found to be potent AChE inhibitors in the μM or nM range.

Wei et al. (2014) prepared uncharged 2-hydroxyiminoimidazoles with peripheral binding ligand (**21**–**22**; Fig. 69.11) and evaluated them on sarin-, VX-, and tabun-inhibited hAChE. One compound (**22**) was found to be effective for sarin-inhibited hAChE better than asoxime, the same compound was comparable with asoxime for VX reactivation and results were comparable with trimedoxime for tabun reactivation in vitro. The novel reactivators were found to be moderate or weak AChE inhibitors in the mM or high μM range.

Wei et al. (2016) published uncharged *o*-hydroxybenzaldoximes (**23**; Fig. 69.11) for reactivation of soman-inhibited hAChE. The restoration of enzymatic activity was determined in various time intervals and was found better than pralidoxime, but far worse than asoxime reactivation ability.

Maraković et al. (2016) designed and prepared uncharged *N*-substituted-2-hydroxyiminoacetamides (**24**–**25**; Fig. 69.12) and screened them on sarin-, cyclosarin-, and VX-inhibited hAChE or hBChE. One compound was found to be beneficial for phosphylated hAChE with reactivation ability over 60% for all OPs and two compounds were found to be promising for phosphylated hBChE with a reactivation ability over 60% for all OPs, but data for a standard reactivator were not provided. The presented compounds resulted as moderate to weak inhibitors of hAChE and strong to moderate inhibitors of hBChE.

Katalinić et al. (2016) published an evaluation of uncharged imidazole or monocharged imidazolium compounds (**26**–**27**; Fig. 69.12) on hBChE inhibited by tabun, paraoxon, or VX. Two molecules were indicated to be able to exceed obidoxime reactivation of paraoxon and five molecules were able to exceed obidoxime and asoxime reactivation of VX. It has to be noted that the majority of the novel molecules were able to strongly inhibit hBChE in a low μM scale.

Wei et al. (2017) developed uncharged conjugates of salicylaldoximes with peripheral site ligands (**28**; Fig. 69.13) that were screened on hAChE inhibited by VX, sarin, tabun, and soman. Four compounds showed an

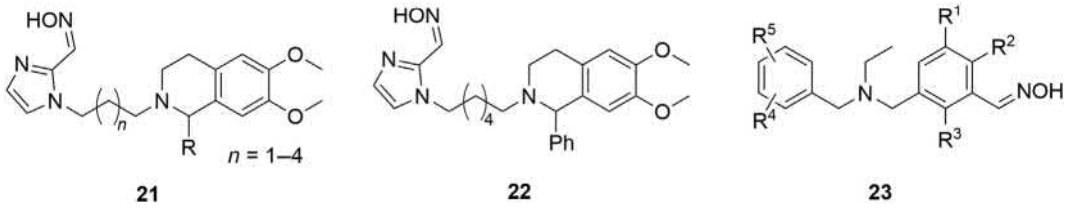


FIGURE 69.11 Uncharged 2-hydroxyiminoimidazoles (Wei et al., 2014; Wei et al., 2016).

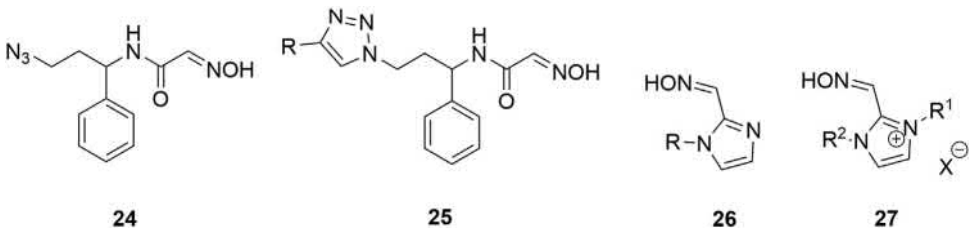


FIGURE 69.12 Uncharged *o*-hydroxybenzaldoximes and *N*-substituted-2-hydroxyiminoacetamides (Maraković et al., 2016; Katalinić et al., 2016).

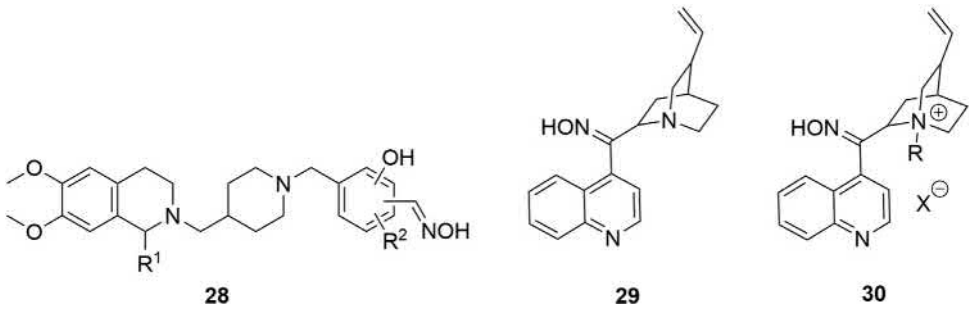


FIGURE 69.13 Salicylaldoximes and cinchona oximes (Wei et al., 2017; Katalinić et al., 2017).

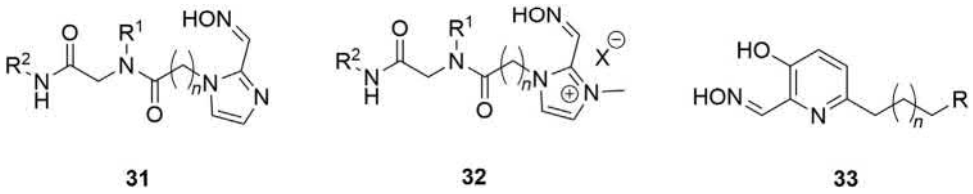


FIGURE 69.14 Uncharged imidazole oximes and 3-hydroxy-2-pyridine aldoximes (de Koning et al., 2017; Zorbaz et al., 2018a).

increased reactivation rate for VX and tabun comparable with obidoxime or asoxime, but none exceeded asoxime for sarin reactivation based on overall reactivation rate constant. The presented salicylaldoximes were moderate or weak inhibitors of hAChE.

Katalinić et al. (2017) published uncharged or monocharged cinchona oximes derived from 9-oxocinchonidine (**29**–**30**; Fig. 69.13) and screened them on sarin-, cyclosarin-, VX-, tabun-, and paraoxon-inhibited hAChE or hBChE. The oximes resulted in weak hAChE reactivators with the best reactivation of ~20% for VX. The better reactivation was observed for hBChE, but all novel compounds were found to be weaker reactivators for tested OPs than standards of asoxime, obidoxime, or K117. Notably, cinchona oximes showed higher inhibition of hBChE in the low μM or nM range.

de Koning et al. (2017) applied an Ugi multicomponent reaction for the synthesis of uncharged imidazole oximes or charged imidazolium oximes with peripheral site ligand (**31**–**32**; Fig. 69.14) that were tested on hAChE inhibited by sarin, cyclosarin, VX, and tabun. For sarin-, cyclosarin-, and VX-inhibited hAChE, the tested oximes were not able to exceed the reactivation ability of asoxime or obidoxime. For the tabun-inhibited enzyme, one compound showed reactivation ability comparable with obidoxime.

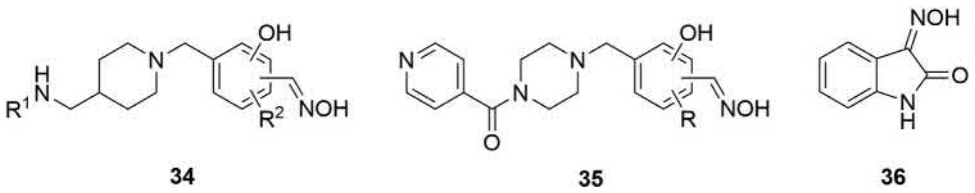


FIGURE 69.15 Salicylaldoximes with peripheral site ligands and isatin-3-oxime (Wei et al., 2018; de Paula et al., 2018).

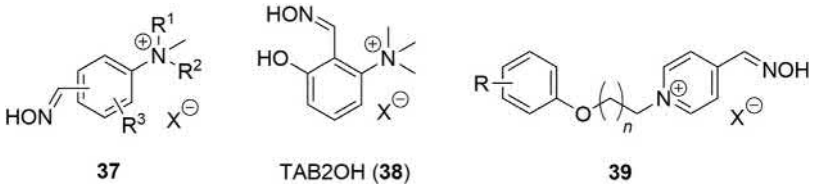


FIGURE 69.16 Trialkylammonium salts with aryloxime and monopyridinium salts with 4-positioned oxime (Radić et al., 2013; Chambers et al., 2013).

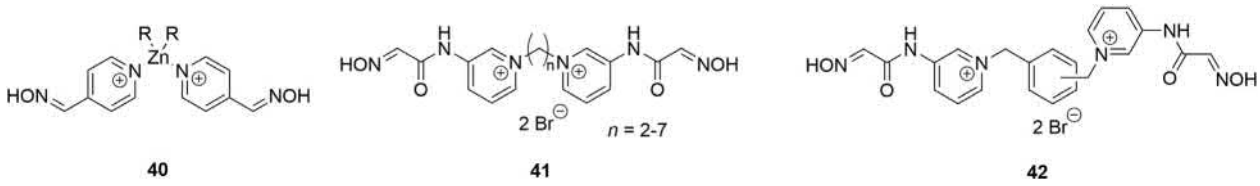


FIGURE 69.17 Zinc complexes of 4-hydroxyiminopyridine and pyridine-3-yl-acetamides (Konidaris et al., 2014; Karade et al., 2014).

Zorbaz et al. (2018a) published uncharged 3-hydroxy-2-pyridine aldoximes (**33**; Fig. 69.14) and evaluated them on hAChE or hBChE inhibited by VX, sarin, cyclosarin, tabun, and paraoxon. The reactivation rates of two oximes for VX-, tabun-, and paraoxon-inhibited hAChE were found to be greater than asoxime. The reactivation rates of several oximes for VX-, cyclosarin-, tabun-, and paraoxon-inhibited hBChE were found to be greater than asoxime. The presented aldoximes were strong to weak inhibitors of hAChE or hBChE.

Wei et al. (2018) designed uncharged conjugates of salicylaldoximes with peripheral site ligands (**34**–**35**; Fig. 69.15) that were evaluated on hAChE inhibited by VX, sarin, tabun, soman, and several OP pesticides. Based on a second-order reactivation rate constant, two compounds were found comparable with obidoxime for tabun reactivation, but they were no more efficient than asoxime for sarin and VX. Some oximes were found to be promising for OP pesticide reactivation when compared to reactivation ability of obidoxime. All compounds resulted as weak inhibitors of hAChE in the mM range.

de Paula et al. (2018) published a study on uncharged isatin-3-oxime (**36**; Fig. 69.15) that was evaluated on paraoxon-inhibited eel AChE. Isatin-3-oxime was found to be worse in reactivation of paraoxon when compared to pralidoxime or obidoxime.

69.8 Mono- or double-charged oxime reactivators

Radić et al. (2013) published trialkylammonium salts with aryloxime moiety (**37**–**38**; Fig. 69.16) for reactivation of paraoxon-, cyclosarin-, VX-, and sarin-inhibited AChE and BChE. The reactivation of AChE relative to pralidoxime was found to be low. The reactivation of BChE relative to pralidoxime identified compound TAB2OH (**38**) with 2-positioned aryloxime and 3-phenolic moiety that was most effective against VX-inhibited BChE.

Chambers et al. (2013) prepared monopyridinium salts with 4-positioned oxime and modified peripheral binding motif (**39**; Fig. 69.16). The compounds were tested on rat brain homogenates inhibited by sarin and VX surrogates (PIMP and NEMP) and their in vitro reactivation ability was found to be lower than pralidoxime or trimedoxime.

Konidaris et al. (2014) synthesized zinc complexes of 4-hydroxyiminopyridine (**40**; Fig. 69.17) and tested their reactivation on paraoxon-inhibited eel AChE. The reactivation ability was found to be far lower than for the reference compound obidoxime. Karade et al. (2014) synthesized a series of double-charged pyridine-3-yl-acetamides (**41**–**42**; Fig. 69.17) and tested them on sarin- and VX-inhibited hAChE. Some of prepared compounds were comparable with pralidoxime and one was

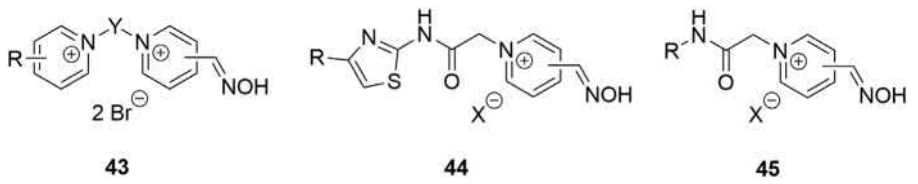


FIGURE 69.18 Double charged oximes tested on hBChE, *N*-thiazolylacetamido pyridinium oximes and pyridinium acetamides (Horn et al., 2015; Valiveti et al., 2015a,b).

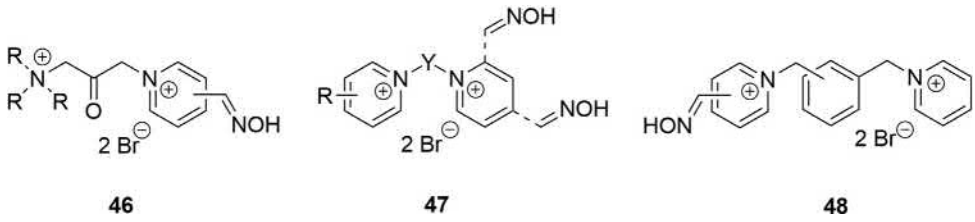


FIGURE 69.19 Monopyridinium oximes with ammonium fragment and double-charged bispyridinium oximes (Bobkova, 2015; Winter et al., 2016; Musilek et al., 2016).

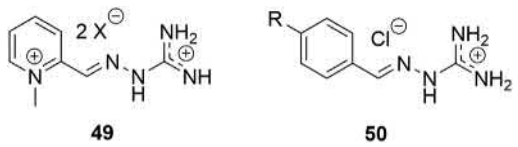


FIGURE 69.20 Monocharged guanylhydrazones (Petronilho et al., 2016).

comparable with obidoxime for sarin reactivation, whereas all novel compounds were found to be worse than obidoxime for VX reactivation concerning the overall reactivation rate constant.

Horn et al. (2015) analyzed the reactivation ability of formerly prepared double-charged oximes (**43**; Fig. 69.18) on BChE inhibited by paraoxon, tabun, and cyclosarin. The tested oximes have a low-to-negligible reactivating potency with paraoxon- and tabun-inhibited hBChE including the commercial oximes (pralidoxime, methoxime, trimedoxime, obidoxime, and asoxime), whereas several novel oximes showed a moderate-to-high potency with cyclosarin-inhibited hBChE. The majority of tested oximes resulted in poor inhibitors of hBChE in the high μM or mM range.

Valiveti et al. (2015a) presented a series of monocharged *N*-thiazolylacetamido pyridinium oximes (**44**; Fig. 69.18) and used them on the model of sarin-, *O*-ethylsarin-, and VX-inhibited hAChE. Concerning the overall reactivation rate constant, the novel oximes were found to be generally worse than pralidoxime and obidoxime for sarin inhibition. For *O*-ethylsarin inhibition, some oximes were comparable in reactivation ability with pralidoxime and one with obidoxime. Two oximes were better than pralidoxime, but none comparable with obidoxime for VX-inhibited AChE.

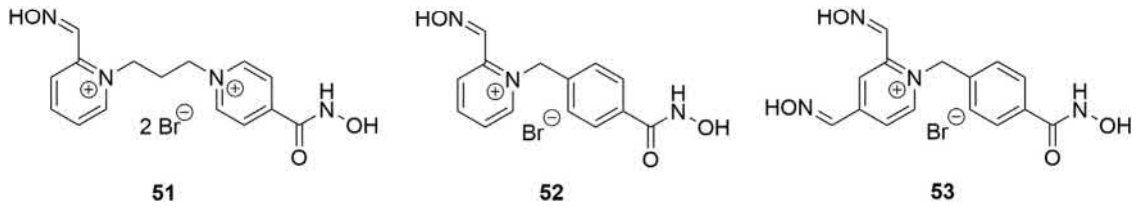
Valiveti et al. (2015b) published a series of monocharged pyridinium acetamides (**45**; Fig. 69.18) which were evaluated on sarin-, VX-, and tabun-inhibited hAChE. Three novel oximes were found comparable and two were better than obidoxime for reactivation of sarin. Additionally, two oximes were found to be comparable with obidoxime for VX reactivation and one oxime for tabun reactivation based on overall reactivation rate constant.

Bobkova (2015) presented a series of double-charged monopyridinium oximes with aliphatic ammonium fragment mimicking choline moiety (**46**; Fig. 69.19) that were tested on VX-inhibited hAChE. Three novel compounds were able to restore hAChE activity better than trimedoxime at varying concentrations.

Winter et al. (2016) tested a large series of double-charged bispyridinium oximes (**47**; Fig. 69.19) on tabun-, cyclosarin-, and paraoxon-inhibited hAChE. Several oximes were highlighted as reactivators for a single OP inhibitor, but no oxime could be rated as a broad-spectrum candidate from this in vitro evaluation.

Musilek et al. (2016) described double-charged bispyridium oximes bridged by xylene moiety (**48**; Fig. 69.19) that were subjected to testing on tabun-, ethylparaoxon-, methylparaoxon-, and DFP-inhibited hAChE. Some novel compounds were found to be better than pralidoxime, but they were not able to exceed the reactivation ability of obidoxime.

Petronilho et al. (2016) prepared monocharged guanylhydrazones (**49**−**50**; Fig. 69.20) as potential inhibitors or reactivators of eel AChE. Some compounds were found to be moderate eel AChE inhibitors, but all failed in reactivation ability when they were compared to pralidoxime.



51 52 53

FIGURE 69.21 Pyridinium oximes with carbamate moiety (Amitai et al., 2016).

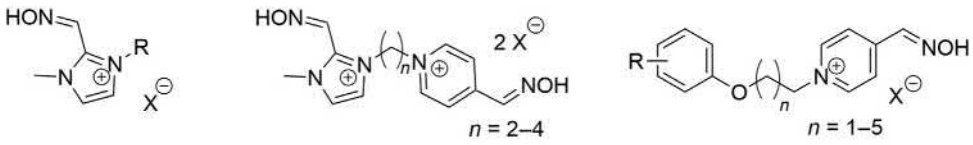


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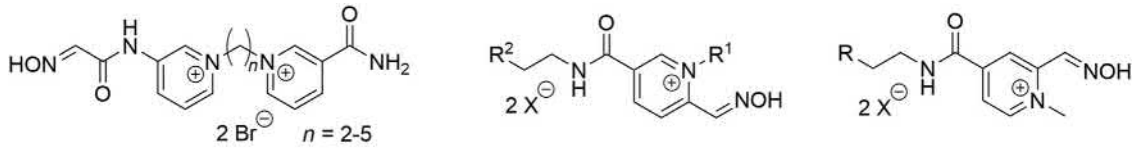
FIGURE 69.22 Imidazolium oximes and phenoxyalkyl pyridinium oximes (Sharma et al., 2016; Chambers et al., 2016).



57 58 59

FIGURE 69.23 Pyridine-3-yl-acetamides and Zinc database oximes (Karade et al., 2016; Berberich et al., 2016).

Amitai et al. (2016) developed a series of bifunctional mono- or double-charged pyridinium oximes with carbamate moiety (**51**–**53**; Fig. 69.21) and tested their reactivation of sarin-, cyclosarin-, and VX-inhibited hAChE. One compound was found to have a better reactivation rate for cyclosarin than pralidoxime, however all compounds were far worse than asoxime for all tested OPs. Novel compounds were proved to be inhibitors of hAChE on a low μM scale, which could be used for prophylactic purposes.

Sharma et al. (2016) prepared monocharged alkylated imidazolium oximes and double-charged imidazolium oximes with second oxime moiety (**54**–**55**; Fig. 69.22) for screening of sarin- and VX-inhibited hAChE. One double-charged compound was found to be better than pralidoxime for sarin reactivation, but all compounds failed to exceed reactivation of obidoxime and asoxime for both OPs. The novel compounds were found to be weak hAChE inhibitors in the mM range.

Chambers et al. (2016) prepared other series of monocharged phenoxyalkyl pyridinium oximes (**56**; Fig. 69.22) and evaluated them on surrogates of sarin- (PIMP) and VX-inhibited (NEMP) bovine brain AChE (bvAChE) in vitro. The compounds were poor reactivators of PIMP-inhibited bvAChE compared to pralidoxime and trimedoxime, which were unfortunately not tested as standards for NEMP-inhibited bvAChE, where the novel compounds provided a maximal reactivation of ~60%. Interestingly, in animal studies, some compounds were found to reactivate rat brain AChE more than pralidoxime in two time intervals.

Karade et al. (2016) designed another series of double-charged pyridine-3-yl-acetamides with pyridinium carboxamide moiety (**57**; Fig. 69.23) which were tested on sarin- and VX-inhibited hAChE. Two compounds were identified to exceed the reactivation ability for sarin-inhibited AChE and one for VX-inhibited AChE better than pralidoxime and obidoxime. The presented compounds resulted as weak inhibitors of hAChE.

Berberich et al. (2016) used virtual screening techniques to identify novel scaffolds from Zinc database and assayed six selected and double-charged compounds (**58**–**59**; Fig. 69.23) for reactivation ability on paraoxon-, DFP-, fenamiphos-, and methamidophos-inhibited hAChE. Five oximes were found to be promising in reactivation of all tested OPs when compared to pralidoxime. Some novel molecules showed a certain level of inhibition to hAChE higher than pralidoxime.

Bušić et al. (2016) prepared monocharged pyridoxal oxime analogs (**60**; Fig. 69.24) that were screened on tabun-, paraoxon-, or VX-inhibited hAChE and hBChE. The reactivation of hAChE or hBChE by novel compounds

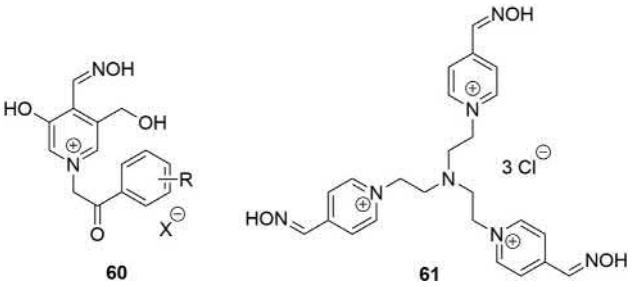


FIGURE 69.24 Pyridoxal oxime analogs and trisoxime (Bušić et al., 2016; Kuca et al., 2016).

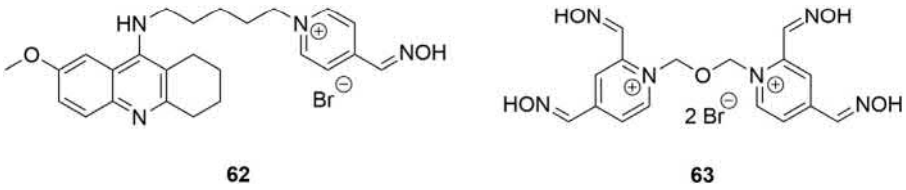


FIGURE 69.25 7-Methoxytacrine-4-pyridinealdoxime hybrid and tetroxime (Nepovimova et al., 2016; Kuca et al., 2017).

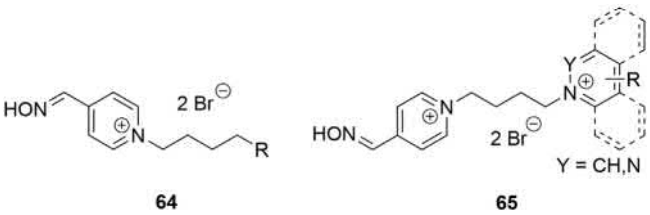


FIGURE 69.26 Double-charged reactivators and analogs of K048 (Kuca et al., 2018; Malinak et al., 2018b).

was found to be low compared to standards trimedoxime, obidoxime, and asoxime. The novel oximes resulted in moderate–high μM inhibitors of both enzymes.

Kuca et al. (2016) presented a triple-charged trisoxime compound (**61**; Fig. 69.24) that was tested on rat brain AChE inhibited by various OP species and compared to monocharged 4-pralidoxime (4-PA) and double-charged K074. The trisoxime reactivated sarin, VX, and DFP better than 4-PA and was comparable to double-charged K048, but remained almost inactive for other OPs.

Nepovimova et al. (2016) designed and prepared a monocharged 7-methoxytacrine–4-pyridinealdoxime hybrid (**62**; Fig. 69.25) for prophylactic purposes and screened it on tabun-, sarin-, VX-, and paraoxon-inhibited hAChE. The hybrid was found to be partially effective in reactivation of all OPs, but generally pralidoxime, 4-PA, or obidoxime. The hybrid was indicated as a potent hAChE inhibitor on a low μM scale.

Kuca et al. (2017) studied a double-charged tetroxime reactivator (**63**; Fig. 69.25) for reactivation of sarin, cyclosarin, VX, and tabun. Tetroxime was a poor reactivator in the majority of OPs when compared to pralidoxime with the exception of VX, where it showed enhanced reactivation ability.

Kuca et al. (2018) presented three double-charged compounds (**64**; Fig. 69.26) that were screened on paraoxon-inhibited hAChE and hBChE. The compounds generally resulted in weak reactivators for hAChE and hBChE compared to pralidoxime, obidoxime, or asoxime.

Malinak et al. (2018b) synthesized double-charged analogs of K048 (**65**; Fig. 69.26) that were tested on tabun-, ethylparaoxon-, methylparaoxon-, and DFP-inhibited hAChE. Several novel compounds were found to be effective reactivators for individual OPs with better ability than used standards, but none was considered to be a broad-spectrum molecule. The inhibitory effect of novel compounds varied from low to high μM levels.

Kim et al. (2018) conjugated tacrine with pyridinium moiety and produced monocharged hybrid oximes (**66**; Fig. 69.27) that were screened on paraoxon-inhibited eel AChE. Some compounds showed reactivation better then pralidoxime and asoxime. The majority of hybrid oximes were found to be strong inhibitors of eel AChE in the low μM range.

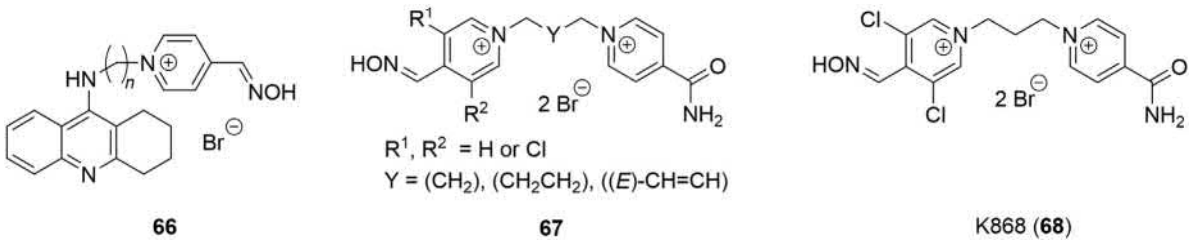


FIGURE 69.27 Tacrine-pyridinium-oximes and chlorinated oximes (Kim et al., 2018; Zorbaz et al., 2018b).

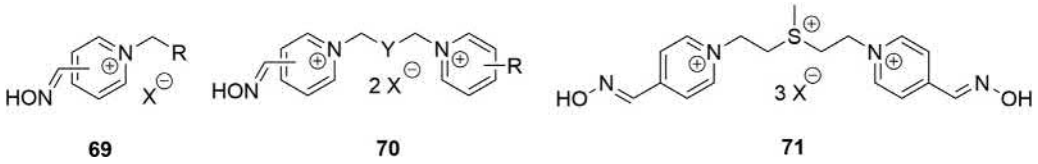


FIGURE 69.28 Monocharged or double-charged pyridinium oximes and triple-charged bisoxime (Bharate et al., 2019; Kuca et al., 2019).

Zorbaz et al. (2018b) designed and prepared double-charge chlorinated oximes derived from K027, K048, and K203 (**67**−**68**; Fig. 69.27) with the aim of enhancing the oximate formation and increasing the nucleophilicity toward the OP-AChE adduct. The chlorinated oximes were tested on hAChE inhibited by sarin, cyclosarin, VX, and tabun. The results showed that novel compounds had enhanced properties in reactivation of all used OPs compared to standards of pralidoxime or even asoxime. The reactivation of tabun was found to be worse than experimental standard K203. Oxime K868 (**68**) was identified to be the most broad-spectrum molecule for reactivation of sarin, cyclosarin, and VX, exceeding the potency of asoxime and also had some tabun reactivation ability (although minor), which is missing in the case of asoxime. The chlorinated oximes were found as medium to weak inhibitors of hAChE in the μM range. Zorbaz et al. (2019) further explored the investigation of chlorinated oximes for hBChE inhibited by sarin, cyclosarin, VX, and tabun for pseudo-catalytic scavenger purposes. Some chlorinated oximes displayed enhanced reactivation ability, namely for sarin- and tabun-inhibited hBChE that was better than pralidoxime or asoxime. Again, oxime K868 was the most broad-spectrum reactivator showing ability to counteract sarin, cyclosarin, VX, and tabun under in vitro conditions.

Bharate et al. (2019) synthesized monocharged or double-charged pyridinium oximes (**69**−**70**; Fig. 69.28) that were subjected to screening on paraoxon-, methylparaoxon-, and DFP-inhibited eel AChE. One novel monocharged oxime was a reactivator comparable in activity with pralidoxime. Two double-charged compounds were shown to reactivate OPs on a similar level as trimedoxime or obidoxime, however their structures were formerly known as K048 and K074.

Kuca et al. (2019) published a study on a triple-charged bispyridinium bisoxime (**71**; Fig. 69.28) that was tested on rat brain AChE inhibited by sarin, cyclosarin, VX, and tabun. The trisoxime was not able to reactivate cyclosarin and tabun, but showed some reactivation of sarin and VX that was rationalized by a molecular docking study.

69.9 In vitro evaluation of selected AChE reactivators

There are many available techniques to evaluate the reactivation activity of structurally different AChE reactivators (Holas et al., 2012). Probably the most frequently used is Ellmann's method (Ellman et al., 1961) which has its advantages and limitations (Sinko et al., 2007). Further, there is variability within the AChE species that can be used for in vitro evaluation. For instance, there is an extensive difference in inhibitory potency of paraoxon with human and guinea pig AChE (Worek et al., 2011), but only moderate differences of the reactivation between human and animal AChE were found. The availability of human AChE/BChE from recombinant sources or from human erythrocytes/plasma has much increased over the last decade. It seems to be important to prefer human sources of enzymes because the data obtained on, for example, eel or rat AChE might be highly different. The interspecies differences are also important for further in vivo evaluation (Herkert et al., 2011; Jaćević et al., 2019).

The tested OP species are related to the decision of the scientific group. Sarin, VX, tabun and OP pesticides are usually and most frequently tested. Sarin is an example of a volatile OP molecule, whereas VX is a representative of persistent OP compounds and tabun is one of the

most difficult reactivable OPs. The surrogates of nerve agents were formerly introduced (Meek et al., 2012; Cavalcante et al., 2019). Their use now allows a broader scientific community to prepare reactivation experiments with the same phosphylated enzymes as with the use of nerve agents. Prior to surrogates, such experiments were available only with low-toxicity OP pesticides (e.g., paraoxon).

Additionally, there are differences in protocols used for cholinesterase activity determination (Maček Hrvat et al., 2018). The results are almost incomparable among various laboratories, however data from one laboratory could be considered to be consistent, comparative, and informative. All results considered in the structure–activity relationship discussed below attempt to compare results obtained in different scientific groups with different methods on different cholinesterase species.

69.10 The structure–activity relationship and discussion

Three major classes of cholinesterase reactivators were developed in the last decade. The uncharged non-oxime reactivators were first described in 2015 and are the smallest group for AChE reactivation, consisting of compounds with various phenol bases. ADOC (**2**) was the most studied compound among them and it was predicted to form quinone methides which are reactive to AChE (de Koning et al., 2018). The aromatic phenol moiety with *o*-positioned tertiary amine hidden in the cycle was found to be the most promising to date. The reactivation ability of such non-oxime compounds was found to some extent to be comparable with pralidoxime, but in most cases it failed to reach reactivation ability of asoxime (Horn et al., 2018).

The uncharged oxime reactivators were being developed for the whole decade. The 3-hydroxy-2-pyridinealdoximes with different peripheral binding site ligands (e.g., tacrine, tryptoline, morpholine) were introduced with very interesting reactivation abilities toward multiple OP species under in vitro conditions (e.g., Zorbaz et al., 2018a). The 3-hydroxypyridine moiety is potentiating the oximate formation and thus reactivation. The peripheral binding site ligands help the molecule to accommodate in the OP-inhibited enzyme, but this structural fragment in some cases led to increased and unwanted cholinesterase inhibitory ability by the uncharged oxime. The in vivo data indicate that the dosing and achieving the appropriate plasma levels for reactivation of uncharged 3-hydroxy-2-pyridinealdoximes might be a problem (Calas et al., 2017).

The hydroxyiminoacetamides (e.g., Kovarik et al., 2013) were designed to be brain penetrable and attainable. There were shown to be very effective in vitro against multiple OP species with a lead structure of RS194B (**4**), but it should be noted that they were usually compared only to pralidoxime and not to more effective standards such as obidoxime or asoxime. However, animal studies revealed the rapid clearance of such small molecules from brain and body (Malfatti et al., 2017), which may limit their further development or use.

The 2-hydroxyiminoimidazoles are another structural group of cholinesterase reactivators which were prepared as uncharged or monocharged compounds (e.g., Wei et al., 2014; Katalinić et al., 2016). The oxime moiety was retained and the binding motif usually consisted of aromatic moiety with different spacer length. Several hit structures with modified aromatic fragments were found to be able to reactivate hAChE or hBChE in vitro. Some potent reactivators were found to have potent and unwanted cholinesterase inhibitors as well.

The *o*-hydroxybenzaldoximes and salicylaldoximes (Wei et al., 2016, 2017, 2018) were designed with phenolic moiety, helping the formation of oximate on the uncharged aromatic ring. The structure was expanded by a peripheral site binding fragment. Some compounds were able to reach reactivation levels of obidoxime or asoxime under in vitro conditions and were found relatively weak hAChE inhibitors. The in vivo data are not available to date. There were also other attempts to design potent uncharged oximes, such as cinchona oximes (Katalinić et al., 2017) or isatin-oxime (de Paula et al., 2018), but with poor reactivation efficiency.

The potential issue for all uncharged oximes is water/buffer solubility for in vivo applicability (Soukup et al., 2018). It has to be noted that reactivators are applied by i.m. injection dissolved in water and the starting dose ranges between 300 and 1000 mg for marketed reactivators. Thus, some level of solubility of uncharged compound in water should be retained for such a high dose, which is necessary to save lives. The in vivo data published to date indicate that this is a crucial issue for uncharged cholinesterase reactivators.

The monocharged and double-charged cholinesterases have been being developed for many decades. The pyridinium or imidazolium fragment with 2- or 4-positioned oxime moiety is usually used for reactivation and pyridinium-connected scaffold serves as an enzyme-binding motif. In some cases, quaternary nitrogen can be pulled out of the aromatic ring (Radić et al., 2013) while retaining a certain level of reactivation ability, or the quaternary ammonium fragment in a double-charged compound can serve as a peripheral binding part of the molecule (Bobkova, 2015). The monocharged compounds with proper peripheral binding motifs were found to have effective reactivation (Chambers et al., 2013), but the double-charged compounds usually have stronger binding

to phosphylated enzymes. However, the addition of quaternary nitrogen to form a triple-charged compound (Kuca et al., 2019) does not bring further reactivation benefit. Similarly, the introduction of strong cholinesterase inhibitors as peripheral binding motifs led in many cases to a decrease in the reactivation ability (Nepovimova et al., 2016; Kim et al., 2018). The addition of another oxime moiety to form bisoxime (e.g., methoxime, trimedoxime, obidoxime), trisoxime (Kuca et al., 2016), or tetroxime (Kuca et al., 2017) may increase the reactivation, although it is not necessary and might complicate the molecule binding in the OP-inhibited enzyme. The single oxime functional group with proper pK_a to form an oximate anion should be sufficient for OP reactivation. Most of the presented charged compounds have pK_a ~7.8−8.5, where oximate formation is limited (Musil et al., 2016). The introduction of chlorine atom(s) in close proximity to the oxime moiety decreased pK_a and helped oximate formation (Zorbaz et al., 2018b). This effect led to increased reactivation of sarin-, cyclosarin-, VX-, and tabun-inhibited hAChE or BChE and was even better than asoxime which serves as the gold standard in the field of cholinesterase antidotes (Zorbaz et al., 2018b, 2019). Thus, oxime K868 (**68**) seems to be a very promising molecule for further research and development.

69.11 Recent trends in the development of new AChE reactivators and future directions

Along with those AChE reactivators that are commercially available (namely, pralidoxime, trimedoxime, methoxime, obidoxime, and asoxime), there are several trends that are seen as promising.

The uncharged nonoxime or oxime molecules became of scientific importance and their reactivation ability under in vitro conditions in many cases reached used standards. However, their research and development have to continue due to unsolved in vivo issues that limit their current use and effectiveness in vivo (Calas et al., 2017; Malfatti et al., 2017; Soukup et al., 2018). Once they have a balanced pharmacokinetic profile, they could serve as brain protection against OP toxicity.

The charged oxime molecules are well known but possibly could be improved. A good example could be oxime K203 that is to date one of best reactivators of tabun-inhibited hAChE (reviewed in Gorecki et al., 2019). There are a few more, for example, K207 and K048, that are undergoing thorough evaluation against OP pesticide intoxications (Antonijevic et al., 2019; Lorke and Petroianu, 2019). More recently, the chlorinated oximes with pK_a-mediated properties emerged from the scientific community as broad-spectrum hAChE reactivators against multiple OP species (Zorbaz et al., 2018b).

Last but not least, the BChE reactivators are extensively being developed for pseudo-catalytic scavenger purposes (Kovarik et al., 2010). Such scavenger could neutralize the OP via BChE (as a stoichiometric scavenger) and further the inhibited BChE could be renewed by an oxime reactivator to be available repeatedly for nerve agent binding. For hBChE reactivation, the chlorinated oximes may be effectively used against multiple OP species (Zorbaz et al., 2019).

69.12 Concluding remarks and future directions

As discussed in this chapter, the threat of misuse of OP nerve agents by terrorist or military fractions continues. The requirements of agricultural production demand the use of OP pesticides, so deaths from these compounds in agricultural workers are increasing. There is no universal reactivator that could be applied in every case of OP nerve agent or pesticide intoxication. Thus, the development of new, effective, and safe cholinesterase reactivators is necessary.

Acknowledgments

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Chapter 70

Paraoxonase (PON1), detoxification of nerve agents, and modulation of their toxicity

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70.1 Introduction

More than 60 years ago, it was discovered that certain organophosphorus (OP) insecticides could be enzymatically hydrolyzed by plasma, and it was determined that this hydrolysis was catalyzed by enzymes which were designated as A-esterases (Mazur, 1946; Aldridge, 1953). Aldridge's proposal that an A-esterase hydrolyzed both phenylacetate and paraoxon (the active metabolite of the OP insecticide parathion), was conclusively proven several decades later, when it was shown that recombinant paraoxonase/arylesterase catalyzed both activities (Gan et al., 1991). Studies in the late 1970s and early 1980s indicated that the plasma hydrolytic activity toward paraoxon was polymorphically distributed in human populations (Playfer et al., 1976; Eckerson et al., 1983; Mueller et al., 1983), suggesting a genetically based differential susceptibility to OP toxicity. The molecular basis of the paraoxonase (PON1) activity polymorphisms (Humbert et al., 1993; Adkins et al., 1993) and their role in the toxicity of OP compounds (Costa et al., 2003a, 2006, 2013; Davies et al., 1996; Li et al., 2000) have been elucidated. Furthermore, novel important roles of PON1 in the metabolism of oxidized lipids, in the bioactivation or detoxification of certain drugs, and in the hydrolysis of quorum-sensing factor, have also emerged, highlighting the multifaceted roles and importance of this enzyme. These latter aspects of PON1 are not discussed in this chapter, but the reader can refer to other publications for further details (Costa and Furlong, 2002; Mackness et al., 2002; Costa et al., 2003b; Draganov and La Du, 2004; Ozer et al., 2005; Ng et al., 2005; Camps et al., 2009; Furlong et al., 2010, 2016).

70.2 PON1 polymorphisms: defining PON1 status

PON1 is a member of a family of proteins that also includes PON2 and PON3, the genes for which are clustered in tandem on the long arms of human chromosome 7 (q21.22). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoproteins (HDLs) (Sorenson et al., 1999; Deakin et al., 2002). PON1 received its name from its ability to hydrolyze paraoxon, its first and most studied OP substrate. PON1 also hydrolyzes the active metabolites of other OP insecticides (e.g., chlorpyrifos oxon, diazoxon), as well as nerve agents such as sarin or soman. However, many other OPs are not hydrolyzed by PON1. Of the three PONs, only PON1 has OP-esterase activity, while all three are lactonases, displaying overlapping but distinct substrate specificities for lactone hydrolysis (Draganov et al., 2005). The crystal structure for a recombinant chimeric PON1 indicates that it is a six-bladed β-propeller, with two calcium ions in the central tunnel, one of which is essential for enzyme activity and the other for structural stability (Harel et al., 2004). However, recombinant PON1 differs from human PON1 in amino acid sequence and OP-esterase activity (Otto et al., 2009; Trovaslet-Leroy et al., 2011), thus the tertiary structure of native PON1 remains to be elucidated.

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Plasma paraoxonase activity in human populations is polymorphically distributed, and individuals with high, intermediate, or low paraoxonase activity can be identified (Eckerson et al., 1983; Mueller et al., 1983; Geldmacher-von Mallinckrodt and Diepgen, 1988; Ginsberg et al., 2009), though, as shown later, the resolution of the three phenotypes is not revealed in an assay with a single substrate (Davies et al., 1996; Richter et al., 2009). Studies in the early 1990s led to the purification, cloning, and sequencing of human PON1 (Gan et al., 1991; Furlong et al., 1991; Hassett et al., 1991), and in the molecular characterization of its polymorphisms (Humbert et al., 1993; Adkins et al., 1993). Two common polymorphisms were observed in the *PON1* coding sequence: a Gln(Q)/Arg(R) substitution at position 192, and a Leu(L)/Met(M) substitution at position 55 (Humbert et al., 1993; Adkins et al., 1993). The gene frequencies of $PON1_{Q192}$ range from 0.75 for Caucasians of northern European origin, to 0.31 for some Asian populations (Brophy et al., 2002). In addition to these two polymorphisms in the coding region of *PON1*, several polymorphisms have been found in the noncoding, 5′ regulatory region of the *PON1* gene, the most significant being the C-108T polymorphism at an Sp1 binding site, with the −108C allele providing levels of PON1 about twice as high on average as the −108T allele (Leviev and James, 2000; Suehiro et al., 2000; Brophy et al., 2001a,b). Complete resequencing of *PON1* from 47 individuals (24 African Americans and 23 Europeans) has revealed more than 160 single nucleotide polymorphisms (SNPs; Furlong et al., 2008). Some of the few that have been characterized so far have allowed reconciliation of discrepancies between PCR analysis of codon 192 and determination of PON1 status (see below; Jarvik et al., 2003)

The L/M polymorphism at position 55 does not affect catalytic activity, but has been associated with plasma PON1 protein levels, with $PON1_{M55}$ being associated with low plasma PON1 (Blatter Garin et al., 1997; Mackness et al., 1998); this appears to primarily result from linkage disequilibrium with the low efficiency of the −108T promoter allele (Brophy et al., 2002), though some data indicate that $PON1_{M55}$ may be somewhat less stable than $PON1_{L55}$ (Leviev et al., 2001; Roest et al., 2007). In contrast, the Q/R polymorphism at position 192 significantly affects the catalytic efficiency of PON1. This polymorphism is substrate-dependent, as the $PON1_{R192}$ alloform hydrolyzes chlorpyrifos oxon and paraoxon more rapidly than $PON1_{Q192}$ in vitro (Li et al., 2000), while both PON1 alloforms hydrolyze diazoxon (Li et al., 2000) and phenylacetate (Furlong et al., 2006) with the same efficiency.

Though most studies investigating the association of PON1 polymorphisms with diseases have examined only the nucleotide polymorphisms (Q192R, L55M, C-108T) with PCR-based assays, it has been argued that measurements of PON1 function (plasma activity) are of greater relevance, as they measure the plasma activity of PON1, which is the most important factor that determines rates of detoxification of both endogenous and xenobiotic substrates. This functional genomic analysis is accomplished using a high-throughput enzyme assay involving two PON1 substrates (either diazoxon and paraoxon or a pair of nontoxic substrates) (Richter et al., 2004, 2008, 2009). This approach, which has been referred to as the determination of the "PON1 status" for an individual (Richter and Furlong, 1999), in addition to providing a functional assessment of the plasma PON_{192} alloforms, also provides the plasma level of PON1 for each individual, thus encompassing the two factors that affect rates at which PON1 detoxifies endogenous and exogenous substrates. Rates of detoxification of compounds that are not affected by the Q192R polymorphism are determined by plasma PON1 concentration. Rates of detoxification of other compounds for which rates are affected by the Q192R polymorphism are determined both by plasma PON1 levels and the PON1 alloform(s) in plasma. In all cases, it is important to determine PON1 plasma levels, and for some compounds the Q192R polymorphism as well. For this reason, many epidemiological studies that have analyzed SNPs alone have not provided useful findings.

For adequate risk assessment, it is important to know PON1 levels and activity. In a given population, plasma PON1 activity can vary up to 40-fold (Eckerson et al., 1983; Mueller et al., 1983; Davies et al., 1996; Richter and Furlong, 1999; Jarvik et al., 2000), and differences in PON1 protein levels up to 13-fold are also present within a single $PON1_{192}$ phenotype (QQ, QR, RR) in adults (Davies et al., 1996). Ample evidence is now available from studies investigating the role of PON1 in cardiovascular disease, that PON1 status (encompassing the Q/R genotype and levels of activity) is a much better predictor of disease than the *PON1* genotype alone (Jarvik et al., 2000; Mackness et al., 2001; Zhao et al., 2012; Bayrak et al., 2012).

70.3 PON1 and the toxicity of OP insecticides

Evidence that PON1 plays a role in modulating the toxicity of OPs has emerged over the past 30 years, though it had been already noted that species differences in PON1 activity correlated with susceptibility to OP toxicity (Brealey et al., 1980; Costa et al., 1987; Furlong et al., 2000). An earlier study by Main (1956) showed that intravenous (i.v.) administration of partially purified PON1 from rabbit serum protected rats from the toxicity of paraoxon. This initial observation was followed by other

studies, which utilized a similar approach; for example, i.v. administration of purified rabbit PON1 to rats protected the animals from the toxicity [indicated by the degree of acetylcholinesterase (AChE) inhibition] of chlorpyrifos oxon and paraoxon (Costa et al., 1990). Additional studies in mice provided evidence that i.v. administration of rabbit PON1 increased serum chlorpyrifos oxonase activity by 30- to 40-fold, and also protected animals against AChE inhibition caused by dermally applied chlorpyrifos oxon (Li et al., 1993). Since i.v. administration of PON1 only increases serum PON1 activity for a short duration, with a half-life of $\sim$6 h, additional experiments investigated whether other routes of administration for PON1 would extend its plasma half-life. When rabbit PON1 was given by the i.v. and i.p. (intraperitoneal) routes, plasma enzyme activity toward chlorpyrifos oxon increased 35-fold and the half-life was extended to 30 h. An even longer half-life, albeit with lower peak activity levels, was found when rabbit PON1 was given by the i.v. and i.m. (intramuscular) routes (Li et al., 1993). These studies also showed that rabbit PON1 could confer protection toward the parent compound, chlorpyrifos, and that protection was also present when the enzyme was given 30 min to 3 h following exposure (Li et al., 1995), suggesting a therapeutic potential for exogenous PON1 in cases of OP poisoning, possibly in combination with other conventional treatments.

In the following years, *Pon1* knockout ($Pon1^{-/-}$) and human *PON1* transgenic mice provided important new tools to investigate the role of PON1 in modulating OP toxicity. Plasma and liver from $Pon1^{-/-}$ mice have no detectable hydrolytic activity toward paraoxon and diazoxon, and very limited chlorpyrifos-oxonase activity (Shih et al., 1998; Li et al., 2000). $Pon1^{-/-}$ mice do not differ from wild-type animals in their sensitivity to demeton-S-methyl, an OP insecticide with a structure similar to malathion, and which is not a substrate for PON1 (Li et al., 2000), but have a dramatically increased sensitivity to chlorpyrifos oxon and diazoxon, and a slightly increased sensitivity to the toxicity of the parent compounds chlorpyrifos and diazinon (Shih et al., 1998; Li et al., 2000). A surprising finding of these studies is that the lack of PON1 did not affect the sensitivity of mice to paraoxon, the substrate for which the enzyme was named, despite the absence of any paraoxonase activity in plasma and liver (Li et al., 2000) (see also below).

When $Pon1^{-/-}$ mice were given exogenous PON1, resistance to OP toxicity was restored (Li et al., 2000). In these studies, purified human $PON1_{Q192}$ or $PON1_{R192}$ was injected, by the i.v. route, into $Pon1^{-/-}$ mice, and the effects of various OPs on brain and diaphragm AChE activity were determined (Li et al., 2000). $PON1_{R192}$ provided significantly better protection than $PON1_{Q192}$ toward chlorpyrifos oxon, a finding also confirmed by a study by Cowan et al. (2001), who administered recombinant adenoviruses containing human *PON1*-LQ or *PON1*-LR genes to BALB/c mice before challenge with chlorpyrifos oxon. Li et al. (2000) also showed that $PON1_{R192}$ and $PON1_{Q192}$ were equally effective in protecting against the toxicity of diazoxon, while neither alloform afforded protection against paraoxon toxicity.

Kinetic analyses of substrate hydrolysis by purified human $PON1_{R192}$ or $PON1_{Q192}$ provided an explanation for the above in vivo findings. The catalytic efficiency of both PON1 alloforms toward chlorpyrifos oxon was very high, with the $PON1_{R192}$ alloform having the highest catalytic efficiency. In the case of diazoxon, catalytic efficiency was still high, though lower than that toward chlorpyrifos oxon; however, there was little difference between the two $PON1_{192}$ alloforms. Though the $PON1_{R192}$ alloform was also much more efficient than the $PON1_{Q192}$ alloform in hydrolyzing paraoxon, its overall catalytic efficiency was very low, and not sufficient to protect against paraoxon toxicity in vivo (Li et al., 2000). This latter finding confirms the hypothesis that PON1 may not degrade paraoxon efficiently at low concentrations in vivo (Chambers et al., 1994; Pond et al., 1995).

Additional experiments were carried out in *PON1* transgenic mice, that is, mice expressing either human PON_{Q192} or human $PON1_{R192}$ on the knockout background (*Tg-hPON1*$_{R192}$ and *Tg-hPON1*$_{Q192}$), or mice expressing the human $PON1_{R192}$ on top of murine PON1. The latter mice, whose serum paraoxonase activity was 3.5-fold higher than wild-type mice, showed similar sensitivity to paraoxon as wild-type mice (Li et al., 2000), further confirming the inefficacy of PON1 for detoxifying paraoxon in vivo. *Tg-hPON1*$_{R192}$ mice were significantly less sensitive to the toxicity of chlorpyrifos oxon and chlorpyrifos than *Tg-hPON1*$_{Q192}$ mice, despite having the same level of PON1 protein in liver and plasma (Cole et al., 2005). Thus, these experiments in transgenic mice confirmed all previous observations. In addition, administration of a recombinant human PON1 variant ($hPON1_{K192}$) to $Pon1^{-/-}$ mice protected them against the toxicity of diazoxon, given either after or before the enzyme (Stevens et al., 2008).

Altogether, these animal experiments indicate that PON1 can modulate the acute toxicity of OPs in a compound-specific manner. In the case of chlorpyrifos oxon, both the level of expression and the Q192R genotype are important determinants of susceptibility, highlighting the importance of assessing PON1 status in potentially exposed individuals. With regard to diazoxon, protection or susceptibility is dictated primarily by the level of expression of PON1, independently of the Q192R genotype, stressing the importance of knowing PON1 levels. Perhaps ironically, PON1 status does not appear to play a role in modulating sensitivity to paraoxon toxicity due to the low catalytic efficiency of paraoxon hydrolysis.

There is limited evidence that PON1 status may also affect susceptibility to OP insecticides in humans. For example, some studies have investigated the role of PON1 in modulating chronic central and/or peripheral nervous system abnormalities, at times referred to as "dipper's flu," associated with exposure of sheep dippers to diazinon. Diazoxonase activity was found to be lower in cases than in controls (Cherry et al., 2002), and sheep dippers in the lowest quintile of serum diazoxonase activity had a greater risk of reporting ill health than those in the other quintiles (Mackness et al., 2003). These and additional findings (O'Leary et al., 2005; Mackenzie Ross et al., 2010; Cherry et al., 2011) suggest that ill health in sheep dippers was more pronounced in individuals who had a lower ability to detoxify diazoxon.

A few other studies are suggestive of a role of PON1 status in modulating susceptibility to various effects of OPs (Lee et al., 2003; da Silva et al., 2008; Lacasana et al., 2010; Singh et al., 2011). However, these studies lack detailed information on exposures, which hampers interpretation of the findings. Exposure to diazinon and to chlorpyrifos, but not to parathion, was found to be associated with an increased risk of Parkinson's disease in carriers of the $PON1_{M55}$ variant (low PON1 activity) (Manthripragada et al., 2010). Exposure to diazinon and chlorpyrifos was also associated with an increased risk of brain tumor in children with low PON1 activity (Searles Nielsen et al., 2005). Finally, a study in pesticide handlers in Washington State, exposed to various OPs (most notably chlorpyrifos), low PON1 catalytic efficiency (Q192), and low plasma activity were associated with increased degrees of plasma butyrylcholinesterase inhibition from baseline levels (Hoffman et al., 2009). A study by Sato et al. (2016) found that PON1 serum activity levels (but not PON1 SNPs) were correlated with urinary metabolites of fenithrothion oxon, chlorpyrifos oxon, and methylchlorpyrifos oxon, in a group of pest control workers.

Overall, human studies available so far provide limited initial evidence that low PON1 status may increase susceptibility to adverse effects of certain OP insecticides. However, further studies are certainly warranted, particularly to ascertain the contribution of PON1 to OP metabolism and toxicity at low levels of exposure.

70.4 PON1 and the toxicity of nerve agents

As said, some OP nerve agents, such as soman, sarin, tabun, and VX, are metabolized in vitro by PON1 (Davies et al., 1996; Rochu et al., 2007; Valiyaveettil et al., 2010). Information is scarce about VX (Peterson et al., 2011), while more information is available regarding the other compounds, particularly soman and sarin. Both sarin and soman are hydrolyzed to a higher degree by $PON1_{Q192}$ than by $PON1_{R192}$ (Davies et al., 1996), and plasma somanase activity and sarinase activity (both in U/L) were found to be 2143 and 335 for QQ homozygotes, and 992 and 38 for RR homozygotes, respectively (Davies et al., 1996). Thus, homozygotes for the $PON1_{Q192}$ allele hydrolyze sarin approximately 10 times better than individuals homozygous for the $PON1_{R192}$ allele, while the ratio is about two for soman (Davies et al., 1996). Results of a kinetic study confirmed these findings; catalytic efficiency for sarin was determined as 0.91 and 0.07 (ratio = 13) for $PON1_{Q192}$ and $PON1_{R192}$ homozygotes, respectively, while values for soman were 2.8 and 2.1 (ratio = 1.3) (Rochu et al., 2007). Soman exists as four stereoisomers (C + P +, C + P−, C−P +, C−P−), with both P− isomers displaying the highest in vivo toxicity; wild-type recombinant human PON1 was shown to stereoselectively hydrolyze soman, with a sixfold overall difference in catalytic efficiency (P + > P−) (Yeung et al., 2007). Further evidence that purified human PON1 and recombinant PON1 can hydrolyze soman and sarin has been provided by Valiyaveettil et al. (2010), who also reported hydrolysis of tabun.

In vivo studies in animals have shown that i.v. administration of naked DNA bearing the human $PON1_{Q192}$ cDNA to mice could elevate plasma somanase activity by about twofold (Fu et al., 2005). The pcDNA/PON1-treated mice survived in greater number and for a longer period after an acute dose of soman (0.2 mg/kg, s.c.) (Fu et al., 2005). More recently, Valiyaveettil et al. (2011a,b) reported that PON1 could protect guinea pigs from the acute toxicity of sarin and soman. In one study, small quantities of purified human or rabbit PON1 were given i.v. to guinea pigs 30 min before exposure to sarin or soman by microinstillation inhalation (846 and 841 mg/m^3, respectively, equivalent to $1.2 \times LC_{t50}$) (Valiyaveettil et al., 2011a). Although there was only a modest increase in plasma PON1 activity, PON1 from both species significantly increased survival time and antagonized several effects of the nerve agents, including AChE inhibition in brain. In a companion study, human recombinant PON1 expressed in *Trichoplusia ni* larvae, was given i.v. to guinea pigs 30 min before exposure to sarin or soman (Valiyaveettil et al., 2011b). Protective effects were essentially identical to those of the other study. In apparent contrast to these findings, a more detailed study by Hodgins et al. (2013) reported that administration of wild-type human PON1 purified from *Trichoplusia ni* larvae (Otto et al., 2010) or induced via adenoviral infection in mice, protected against the toxicity of the OP insecticides diazoxon and chlorpyrifos oxon, but not of several nerve agents (tabun, soman, sarin, cyclosarin). These findings re-emphasize the fact that human PON1 has a low catalytic activity toward nerve agents and suggest that there is

the need for developing novel human PON1 mutants with enhanced nerve agent-hydrolyzing activity. These studies also suggest that the *Pon1*$^{-/-}$ mouse may be a much better model than the guinea pig for two reasons: (1) there is no background OP hydrolase activity in the plasma of *Pon1*$^{-/-}$ mice, allowing for the pharmacokinetics to be clearly delineated; and (2) much less PON1 (purified or recombinant) is required to raise the plasma level of mice to quite high levels of activity (Li et al., 1993, 1995, 2000; Stevens et al., 2008).

There are different approaches for engineering recombinant PON1 variants with higher catalytic efficiency for treating OP exposures, which represents a major area of current research. One initiated by Tawfik and colleagues begins with the chimeric PON1 that they engineered for solubility and the determination of the crystal structure (Harel et al., 2004). The goal of this approach is to make the sequence of variants with high catalytic efficiency of agent hydrolysis (Aharoni et al., 2004) more "human-like," in order to minimize immunogenicity (Sarkar et al., 2012). A second approach is to begin with the native human rPON1 and introduce the minimal number of mutations required for the desired catalytic efficiency (Stevens et al., 2008). These two approaches are complementary and inform each other. Indeed, Ashani et al. (2011) found that several recombinant PON1 variants were capable of detoxifying cyclosarin in vitro, and similar findings were reported by Goldsmith et al. (2012), whose evolved PON1 had a 340-fold higher efficiency toward cyclosarin. These and other recombinant PON1s were also capable of protecting against OP nerve agent toxicity in vivo (Worek et al., 2014; Ashani et al., 2016; Mata et al., 2016).

The 1995 terrorist attack in the Tokyo subway system that left 12 people dead and over 5000 injured (Suzuki et al., 1995; Nagao et al., 1997) provided the opportunity to investigate the role of PON1 in modulating the toxicity of sarin in humans. The prevalence of the *PON1*$_{R192}$ genotype in the Japanese population is 0.66, compared with 0.25−0.30 in various Caucasian populations (Brophy et al., 2002; Yamasaki et al., 1997). Thus, Japanese individuals may have been more prone to sarin toxicity because of the low sarin-hydrolyzing ability of the PON1$_{R192}$ allozyme. However, among 10 of the victims of the Tokyo attack, seven expressed the *PON1*$_{192Q}$ genotype, with six Q/R heterozygotes and one Q/Q homozygote (Yamada et al., 2001). Thus, the genotype which confers high hydrolyzing activity toward sarin did not appear to provide protection from acute sarin poisoning. Several issues need, however, to be considered. First, only the Q192R genotype of those 10 individuals was analyzed, with no information on their PON1 status. In a Caucasian population, the range of sarinase activity among individuals with the QQ or QR genotype ranged from ∼0 to 758 U/L (Davies et al., 1996). Second, exposure to sarin in these seven QQ or QR individuals was indeed massive, as it caused death instantly or, with one exception, in less than 48 h (Yamada et al., 2001). Such high-dose exposure would be expected to overcome any potential protection afforded by the *PON1*$_{Q192}$ genotype. Third, and most importantly, the catalytic efficiency of sarin hydrolysis by PON1, even in QQ homozygotes, is low; the situation is thus similar (albeit reversed) to that of paraoxon, with one PON1$_{192}$ alloform hydrolyzing sarin with better efficiency, but still not sufficiently high enough catalytic efficiency to provide protection against nerve agent exposure.

A few studies have also investigated PON1 polymorphisms in US and UK troops that were deployed in the Persian Gulf area in 1990−91. Individuals who served in the Gulf War theater were potentially exposed to a wide range of biological and chemical agents, including sand, smoke from oil well fires, solvents, petroleum fuels, depleted uranium, anthrax and botulinum toxoid vaccinations, insecticides, pyridostigmine bromide, and nerve agents (IOM, 2000, 2003). Many of these veterans have complained of a range of unexplained illnesses including chronic fatigue, muscle and joint pain, loss of concentration, forgetfulness, and headache, symptoms that are often referred to as Gulf War syndrome (IOM, 2000, 2003). PON1 genotypes and plasma enzyme activity were investigated in a group of 25 ill Gulf War veterans and 20 controls (Haley et al., 1999). PON1$_{R192}$ homozygotes or PON1$_{Q/R192}$ heterozygotes were more likely to have neurologic symptoms than individuals homozygous for PON$_{Q192}$. Furthermore, low activity of the plasma PON1$_{Q192}$ isoform appeared to better correlate with illness than the PON1 genotype or the activity levels of the PON1$_{R192}$ genotype (Haley et al., 1999). This study would suggest that low PON1 status may represent a risk factor for illness in Gulf War veterans, though such findings necessitate further confirmation in a larger population (Furlong, 2000).

A similar study in a group of 152 UK Gulf War veterans, who self-reported the presence of symptoms associated with Gulf War syndrome, yielded somewhat different results (Mackness et al., 2000). Plasma paraoxonase activity and levels of PON1 protein were lower in veterans than in a control group, and these decreases were independent of the *PON1* genotype (Mackness et al., 2000). Thus, while in both studies a reduced plasma paraoxonase activity was found, in one case it was attributed to an overrepresentation of the low-activity PON1 isozyme (Haley et al., 1999), whereas in the other it was common to all *PON1* genotypes (Mackness et al., 2000). Though the latter study suggests that this group of veterans may have a decreased capacity to hydrolyze some OP insecticides, such as chlorpyrifos oxon, its significance is

hampered by the lack of information on the extent of exposure to such compounds among veterans (Costa et al., 2003a). An additional study compared *PON1* genotypes and plasma paraoxonase activity in groups of UK veterans from the Persian Gulf War who were symptomatic by self-reporting ($n = 115$), healthy Persian Gulf War veterans ($n = 95$), symptomatic Bosnia peacekeeping veterans ($n = 52$) and symptomatic nondeployed military controls ($n = 85$) (Hotopf et al., 2003). No differences in genotype distribution or PON1 activity were found between healthy and ill Gulf War veterans. However, individuals who were deployed to the Gulf had 25%–35% lower median PON1 values than the other two groups, and these differences were not explained by differences in $PON1_{192}$ genotypes between groups. Thus, $PON1_{192}$ genotype and activity were not associated with Gulf War syndrome but appeared to be the results of deployment in the Persian Gulf. Possible explanations suggested for such findings were the potential exposure of those who served in the Gulf to yet unknown agents which led to a long-term decrease in PON1 activity, and/or an overrepresentation in those two groups of individuals with the −108T allele, which is associated with lower PON1 levels (Hotopf et al., 2003). A study by Concato et al. (2007) found no differences in activities of paraoxonase and arylesterase between symptomatic and asymptomatic veterans of the first Gulf War. More recently, a lack of association between PON1 activity and genotypes and neurologic symptom complex or posttraumatic stress disorder in Gulf War era US veterans was also recently reported (Haines et al., 2017).

70.5 PON1 as a therapeutic agent

In recent years, there has been much interest in developing new means of antagonizing the toxicity of OPs, particularly of nerve agents (Masson and Rochu, 2009; Nachon et al., 2013). Current therapy for OP poisoning relies on the use of atropine, a cholinergic muscarinic antagonist, and on oximes, such as pralidoxime (2-PAM), to reactivate phosphorylated acetylcholinesterase before "aging occurs" (Lotti, 2000; Eyer et al., 2007). Anticonvulsant drugs such as diazepam may also be utilized to control OP-induced convulsions (Lotti, 2000). More efficacious pyridinium 4-aldoximes, such as obidoxime and trimedoxime, have also been developed; however, their ability to reactivate phosphorylated acetylcholinesterase is not fully exploited because of the formation of phosphoryloximes, which have themselves very high anticholinesterase activity. Interestingly, phosphoryloximes are rapidly hydrolyzed by PON1, with the $PON1_{Q192}$ allozyme being about 50-fold more active than $PON1_{R192}$ (Stenzel et al., 2007), suggesting that PON1 status would also influence the effectiveness and safety of these oximes (Eyer et al., 2007).

While current therapies are effective in preventing lethality, it has been suggested that they may not prevent behavioral deficits, incapacitation, loss of consciousness, or the potential for permanent brain damage caused by OP nerve agents (Lenz et al., 2007). Complementary approaches have thus focused on the use of human proteins that would act as biological scavengers for OP compounds. Such biological scavengers should have no effects on their own, particularly should not present an antigenic challenge to the immune system, should act rapidly and specifically, and should remain in circulation for a prolonged period (Lenz et al., 2007; Suzuki et al., 2010). Out of this general concept, two parallel approaches have emerged, one relying on stoichiometric, the other on catalytic, bioscavangers. Among stochiometric bioscavengers, B-esterases, such as acetylcholinesterase and butyrylcholinesterase, which react with OPs but do not catalyze their hydrolysis, have been utilized in several studies. Administration of these two proteins to rodents has been shown to afford protection toward two- to fivefold LD_{50} doses of sarin, soman, or VX (Wolfe et al., 1987; Doctor and Saxena, 2005; Lenz et al., 2007; Saxena et al., 2011). Of interest in this regard is human butyrylcholinesterase, either isolated from human plasma, or recombinant (Cerasoli et al., 2005).

While stoichiometric scavengers may offer some protection toward OP toxicity, high doses are needed to neutralize an equimolar amount of nerve agent (Ashani and Pistinner, 2004; Valiyaveettil et al., 2012). In contrast, a catalytic scavenger would afford similar or even higher protection at relatively low doses, as one molecule of a catalytic scavenger can hydrolyze a very large number of molecules of an OP (Sweeney and Maxwell, 2003; Stevens et al., 2008; Suzuki et al., 2010; Valiyaveettil et al., 2012; Worek et al., 2016; Masson and Lushchekina, 2016; Masson and Nachon, 2017; Goldsmith and Ashani, 2018). For example, it has been estimated that 3 μM sarin would be neutralized by 765 mg of human butyrylcholinesterase, but only 120–550 mg of $huPON1_{Q192}$ (depending on time) (Rochu et al., 2007), or a much lower dose of engineered recombinant human PON1 with significantly increased catalytic efficiency of agent hydrolysis. Several arguments would support the use of PON1 as a catalytic bioscavenger (Nachon et al., 2013). PON1 is a human protein, suggesting the possible absence of an immunological response. However, reports of PON1-induced cytotoxicity in macrophages need to be further investigated (Mackness and Mackness, 2010). Experiments in mice and guinea pigs have clearly shown that administration of purified human PON1 or of recombinant PON1 protects animals against the toxicity of specific OP compounds, provided that the

catalytic efficiency is sufficient (Li et al., 2000; Cowan et al., 2001; Stevens et al., 2008; Suzuki et al., 2010; Valiyaveettil et al., 2011a,b; Duysen et al., 2011; Hodgins et al., 2013). In this regard, novel ways to deliver PON1 are being investigated, in addition to administration of purified enzyme (e.g., Li et al., 2000) or of an adenoviral vector (e.g., Cowan et al., 2001; Duysen et al., 2011; Hodgins et al., 2013). For example, Gaidukov et al. (2009) showed that administration of BL-3050, a highly stable engineered human PON1−HDL complex, protected mice against the toxicity of chlorpyrifos oxon, and did not cause any apparent adverse effect on its own. In another study, human PON1 was re-engineered as an IgG−PON1 fusion protein, in which the 355 amino acid human PON1 was fused to the carboxyl terminus of the heavy chain of a chimeric monoclonal antibody against the human insulin receptor (Boado et al., 2011). This fusion protein, designated as HIR-Mab-PON1, was able to enter the brain when injected i.v. into nonhuman primates. This would be of much interest as PON1 is basically absent from brain cells (Giordano et al., 2011), while OPs easily penetrate the blood−brain barrier.

PON1 has been shown to hydrolyze soman, sarin, and tabun, though its catalytic efficiency toward OP nerve agents, in particular sarin, is low. It has been calculated that to provide a valuable medical countermeasure against intoxication by nerve agents, the catalytic efficiency of PON1 will need to be enhanced by one or two orders of magnitude (Rochu et al., 2007). Some direct evolution variants of a recombinant PON1 were found to exhibit a 10-fold faster detoxification of cyclosarin and soman (Amitai et al., 2006). Other huPON1 mutants with enhanced catalytic activity have also been described, though not in relationship to OP nerve agents (Yeung et al., 2004). Directed evolution of a chimeric PON1 made via mammalian gene shuffling, combined with high-throughput screening, led to an evolved variant of chimeric PON1 with a 100,000-fold higher catalytic efficiency toward the most toxic enantiomer of a coumarin analog of cyclosarin (Gupta et al., 2011). However, chimeric PON1 is expected to be immunogenic, hence its practical application is doubtful. Nevertheless, further efforts to develop human PON1 variants with increased catalytic efficiency toward nerve agents are warranted.

An additional approach would be that of stimulating the biosynthesis of natural PON1. Several compounds have been shown to stimulate the expression of PON1 (Costa et al., 2005, 2011). Among them are low doses of ethanol, some fibrates, statins, or dietary polyphenols such as quercetin (Gouedard et al., 2004a). The red wine ingredient resveratrol has been shown to increase PON1 activity nearly threefold in vivo (Gouedard et al., 2004b) and less than twofold in liver cells in vitro (Curtin et al., 2008). Interestingly, in the latter study, the increased PON1 afforded protection of cells against the toxicity of soman and sarin (Curtin et al., 2008). Modulation of PON1 activity by drugs or dietary agents is of interest, though more information of the regulation of the *PON1* gene is needed to devise approaches that would offer substantially larger increases in PON1. However, it has been also argued (Valiyaveettil et al., 2012) that a moderate increase of PON1 (less than twofold) may perhaps be sufficient to provide some protection against some of the symptoms of OP exposures, as shown by the protection seen against sarin and soman in guinea pigs (Valiyaveetil et al., 2011a,b). Nevertheless, as said, the low catalytic efficiency of nerve agent hydrolysis by native human PON1 may not be adequate to provide efficient protection against nerve agent exposure. The recent report by Hodgins et al. (2013) supports the point that the catalytic efficiency for hydrolysis of a specific OP must be sufficiently high to protect against exposure to that specific OP. Work by Chambers et al. (2015) appears to support these conclusions. These investigators identified a few small nucleophilic molecules which are capable of increasing PON1-mediated detoxification of a surrogate of sarin (nitrophenyl isopropyl methylphosphonate) and a surrogate of VX (nitrophenyl ethyl methylphosphonate) in vitro. When tested in vivo in rats, these molecules reduced the degree of inhibition of AChE by the sarin and the VX surrogates (Meek et al., 2015), further indicating that strategies aimed at increasing PON1-mediated detoxification in vivo may provide additional protective action in conjunction with existing therapeutic approaches.

70.6 Concluding remarks and future directions

Evidence from in vitro studies shows that PON1 can hydrolyze several OP compounds, including nerve agents. In vivo studies in rodents have also indicated that PON1 status can influence the acute toxicity of certain OPs, and that administration of exogenous PON1 can protect animals from OP toxicity. In the case of nerve agents, the catalytic efficiency of PON1 is relatively low, and PON1 status does not greatly influence an individual's susceptibility to their toxicity. However, recombinant variants can be engineered with enhanced catalytic activity toward nerve agents. The nonglycosylated, engineered recombinant PON1s with high catalytic efficiency for OP hydrolysis should be excellent candidates for use as catalytic biological scavengers, with both prophylactic and therapeutic applications. As previously suggested (Suzuki et al., 2010; Costa et al., 2011; Hodgins et al., 2013), efforts in this direction and those attempting to increase endogenous PON1 should proceed in parallel, as a combined strategy may prove successful. The increase in

endogenous PON1 levels should be efficacious for exposures to the OPs known to be hydrolyzed at rates sufficient to provide protection (Li et al., 2000; Hodgins et al., 2013). This line of research would have important practical relevance with regard to nerve agents, as shown by recent evidence of the proven use of sarin in Syria in 2013 (John et al., 2018).

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Chapter 71

The role of carboxylesterases in therapeutic interventions of nerve agent poisoning

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71.1 Introduction

Carboxylesterase, sometimes abbreviated as CarbE and sometimes, more recently, as CE, comprises an important class of hydrolytic enzymes and has the Enzyme Commission number EC 3.1.1.1. Aldridge (1953) proposed a division of all esterases into three groups: A-esterases that can metabolize organophosphorus compounds (OPCs) without being inhibited by them (Walker and Mackness, 1987), B-esterases that can simply bind and hence inactivate OPCs by removing them from blood, and C-esterases that do not react with OPCs. B-esterases include carboxylestrases (CarbE, EC 3.1.1.1), acetylcholinesterase (AChE, EC 3.1.1.7), cholinesterase (ChE, EC 3.1.1.8), and some other esterases.

Mammalian CarbEs constitute a large multigene family of α,ß-hydrolase-fold proteins involved in the metabolism of lipids, endogenous fatty acids esters, steroids, and a large number of ester-containing drugs and prodrugs, such as salicylates, clofibrate, procaine, lorazepam, cilazapril, and other angiotensin-converting enzyme inhibitors, narcotics (cocaine, heroin), capsaicin, statins, antiplatelet drugs, immunosuppressants, anticancer drugs, and antiviral agents. CarbEs also participate in detoxification of pesticides (carbofuran, pyrethroids, OPs), acrylates, mycotoxins (T2 toxin), and esters of nicotinic acid (Jokanović, 2015; Laizure et al., 2013). Of five main groups of mammalian CarbE (Satoh and Hosokawa, 1998), two, CE1 and CE2, are the most important ones in humans, with hCE1 predominantly found in the liver and hCE2 in the intestines (Laizure et al., 2013).

71.2 Enzymology of carboxylesterase

CE1 group of CarbE is the largest of the five and contains liver CarbEs of mice, rats, nonhuman primates, and humans. The influence of species on the substrate-specificity is significant and there are also within the same species more than one CarbE isoenzymes, for example, two in rat plasma and three in guinea pig plasma (Sterri and Fonnum, 1987, 1989).

All B-esterases have an active serine-moiety at their active centers that directly interacts with a molecule of an OPC. AChE, ChE, and CarbE contain a "catalytic triad" containing serine, histidine and glutamate—Ser_{200}—His_{440}—Glu_{327} (or aspartate) (Soreq et al., 1992; Cygler et al., 1993; Bajgar, 2005). Their active site is located, at least when AChE is concerned, at the bottom of a 20 Å-deep and 5 Å-wide gorge (Å = ångström = 10^{-8} m), with its walls lined with multiple conserved aromatic residues (Cheng et al., 2017). The specificity of CarbE differs from AChE and ChE in active site volume, electrostatic character, and cation-pI binding. These three enzymes all have a gorge at the active site, however, it varies in size (Sterri and Fonnum, 2015). It appears that AChE has the smallest gorge (296 D3), ChE somewhat larger (496 D3), while CarbE, has by far the largest (3014 D3 or 10 times the size of the gorge of AChE). This explains why diisopropyl fluorophosphate (DFP) cannot inhibit AChE, but it can inhibit ChE and CarbE, since the DFP molecule is too large for AChE and compliant with the ChE and CarbE gorges. In addition, ChE has seven anionic groups reacting with cationic substrates, whereas CarbE has only two. Further,

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AChE has 14 aromatic residues lining the active site gorge, ChE has 7, and CarbE has only 2 (Ripoll et al., 1993; Shafferman et al., 1994). This explains the attraction of VX for AChE and ChE, but not for CarbE (Jokanović, 2001; Sterri and Fonnum, 2015). An additional factor that contributes to the weak reaction of VX with CarbE is its positive charge at phosphorus atoms (Jokanović, 2001).

The steps for CarbE interacting with OPC are the following:

1. The P─F bond (P−N in the case of tabun) of the OPC is attacked by the OH-group of serine.
2. The hydrogen bond between the negatively charged oxygen of a tetrahedral complex and the NH groups of glycine is stabilized by the charged phosphate oxygen of the inhibitor.
3. The P─F bond (P−N in the case of tabun) breaks and as a consequence the leaving group (F, or N in the case of tabun) diffuses away from the active center.
4. A water molecule attacks the serine-*O*-phosphoryl group on the enzyme. Then, histidine delivers a hydrogen atom to the serine-*O*-phosphoryl, which releases the phosphoryl group (Sterri and Fonnum, 2015).

A similar process happens in the case of AChE, with the difference that before the last step an intermediate reaction happens and the OPC undergoes a process known as dealkylation that makes it impossible for the phosphoryl group to leave the active center of AChE, thus rendering the enzyme permanently inhibited and resistant to asoxime (HI-6)-induced dephosphorylation (Fleisher and Harris, 1965; Worek et al., 2018). It is usually said for this dealkylated state of the enzyme−inhibitor complex that the complex was aged and hence the process described is called "aging."

The rate of aging differs when it comes to various OPCs, with soman being dealkylated with a half-time of only 2.3 min (Fleisher and Harris, 1965). Aging half-times ($t_{1/2}$) for tabun, sarin, soman, and VX are of 46 h (de Jong and Wolring, 1978), 5 h (Sidell and Groff, 1974), 1.3 min (Harris et al., 1978), and 48 h (Sidell and Groff, 1974), respectively. When human AChE is taken into account, under the same experimental conditions in human hemoglobin-free erythrocyte ghosts in vitro, the aging half-times (expressed in hours) for tabun, sarin, soman, and VX are: 19.0, 3.0, 0.04, and 36.5, respectively (Worek et al., 2004; 2005). The "aging" process does not happen in the case of CarbE, due to:

1. Different position of histidine within the active site gorge—once it is attached to the active center of the enzyme, the alkoxy group of the soman residue is farther from the histidine group (7Å in CarbE vs. 3.5 Å in AChE) (Maxwell and Brecht, 2001).
2. Very rapid spontaneous reactivation of CarbE inhibited with sarin, soman, and dichlorvos. This issue was discussed in more detail by Jokanović (2015) and in Chapter 55 on biotransformation of organophosphorus compounds in this book.

71.3 Carboxylesterase reactivation

There is a significant level of explicitness in the specificity of nerve agents for inhibition of various B-esterases. Sarin and soman have a roughly equal affinity for inhibition of all three enzymes (AChE, ChE and CarbE), while DFP equally inhibits ChE and CarbE and practically does not inhibit AChE at all (with 1000-fold less affinity than ChE and CarbE). At the same time, VX inhibits AChE 10,000-fold greater than CarbE. As mentioned earlier, the complex AChE−nerve agent ages at certain rates that depend on the inhibitor, while it does not happen with the CarbE−nerve agent complex; it even goes through hydrolysis and the CarbE molecule is recovered and ready to bind to another molecule of nerve agent. It should be noted however that the half-times of the spontaneous reactivation of CarbE is rather slow—2 h for sarin, 20 h for paraoxon, and 40 h for DFP (Jokanović, 2001, 2015; Sterri and Fonnum, 2015). Polak and Cohen (1969) have shown in rats that sarin-induced inhibition of plasma CarbE activity amounts to 50%−60% and disappears 24 h after poisoning. CarbE activity becomes reactivated even after soman poisoning—in guinea pigs, 1 h after poisoning with 0.5 median lethal dose (LD_{50}) of soman the inhibition of CarbE activity was 70%, while it became fully restored after 24 h. As a consequence, repeated administrations of sublethal doses of soman in guinea pigs led to tolerance of 5−6 acute LD_{50} of soman (Sterri et al., 1981). The same mechanism exists in the case of daily subacute intoxication of rats with 0.1−0.4 LD_{50} of tabun, where animals survived until the end of the experiment, that is, until day 28 (Stojiljković et al., 2004).

It was shown that some oximes and hydroxamic acids, like diacetylmonoxime (DAM) and monoisonitrosoacetate (MINA), can protect against sarin poisoning (Askew, 1956, 1957). DAM cannot effectively reactivate AChE, but can increase the rate of reactivation of nerve agent-inhibited CarbE. This effect of DAM is mostly present in plasma and less so in the brain, erythrocytes, and lungs of rats and mice (Myers, 1959). It is interesting that DAM caused a 50%−60% reactivation of sarin-inhibited plasma CarbE in vitro within 5 min, however this level of CarbE activity remained unchanged until 30 min (Sterri and Fonnum, 1987, 1989).

71.4 Source and induction of carboxylesterase activity

The sources of plasma CarbE are considered to be the liver and intestines (Yan et al., 1995; Sterri, 1989). Pretreatment of rats with phenobarbital 100 mg/kg i.p. for

3 days resulted in induction of CarbE activity, but significantly more in the liver (by 56%−77%) than in plasma (by 14%−22%) (Sterri et al., 1985b). In a separate experiment a 4-day phenobarbital (100 mg/kg) pretreatment in mice did not result in changes of AChE activity but resulted in 54% and 48% increases in the plasma and liver CarbE activity, respectively (Clement, 1983). In a similar experiment, 5-day-long pretreatment of rats with phenobarbital 60 mg/kg i.p. resulted in 78% and 85% increases of CarbE activities in plasma and liver, respectively (Jokanović, 1989).

It was shown that treatment of rats with propylthiouracil for 2−3 weeks can induce a hypothyroid status (Stakkestad and Bremer, 1983). Based on that, it was shown that hypothyroidism induced by feeding rats with rat chow supplemented with thiouracil 0.5% for 21 days resulted in increases in plasma BuChE and CarbE activity, by 79% and 89% in comparison with the controls, with accompanying decreased sensitivity to soman toxicity (Swisher et al., 1986). In separate experiments (Sterri and Fonnum, 2015), the effects of hypothyroidism induced by thiouracil in female and male rats, on the activity of ChE and CarbE in plasma and liver were studied (Fig. 71.1).

The esterase activities registered with ethyl butyrate as a substrate were significantly increased only in liver but not in plasma of either male or female rats, while the results obtained with 4-nitrophenyl butyrate as a substrate were inconsistent. In the same animals ChE activities of plasma and liver decreased in females and increased in males, which is in accordance with the notion that ChE activity in rat plasma and liver are sex-dependent (Leeuwin, 1966; Andersen et al., 1983; Edwards and Brimijoin, 1983; Sterri et al., 1985a). These results do not support the notion that plasma CarbE are synthesized by the liver, as it was proven that plasma ChE is undoubtedly synthesized in the liver (Augustinsson, 1948; Koelle, 1963). On the contrary, plasma CarbEs are more likely to originate from the intestine, since the two enzymes share similarly low isoelectric points (pI) of 4.0−4.8, as opposed to the 5.0−6.4 pI range of the six liver CarbE isoenzymes (Mentlein et al., 1987; Sterri, 1989). In addition, it was shown that two CarbE isoenzymes in plasma of both rats and guinea pigs and the three isoenzymes from rat small intestines can be reactivated by DAM after inhibition with soman (Sterri and Fonnum, 1987, 1989; Sterri, 1989), while all three guinea pig liver isoenzymes cannot (Sterri and Fonnum, 1987).

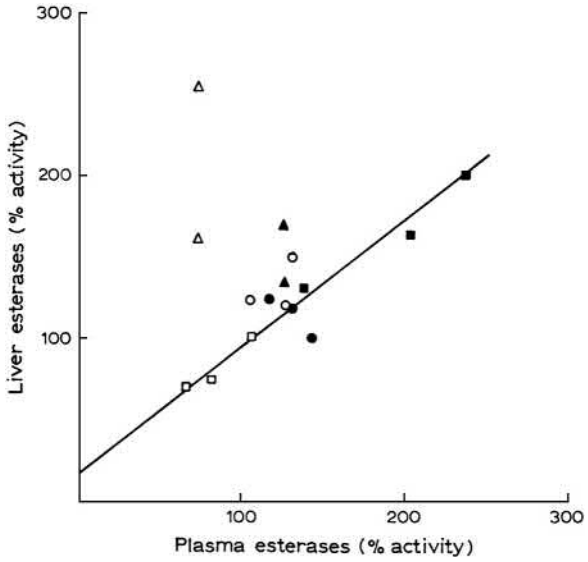

FIGURE 71.1 CarbE and ChE activities in liver versus blood plasma of thiouracil-fed rats. The animals received rat chow supplemented with 0.05% thiouracil for 22, 30, or 38 days. Methyl butyrate hydrolysis (▲△) and 4-nitrophenyl butyrate hydrolysis (●○) were measured in liver homogenate and plasma in accordance with Sterri et al. (1985b), and AChE hydrolysis (■□) by the method of Sterri and Fonnum (1978). The activities are percent of corresponding control (=100%) activities in male (*closed symbols*) and female (*open symbols*) animals fed standard rat chow.

71.5 Carboxylesterases as scavengers of nerve agents

Earlier, it was recognized that plasma CarbE represents a depot for binding sarin or soman that can be reactivated in order to decrease the nerve agent toxicity (Myers, 1959). The mechanism involves protection of AChE, not only in blood, but also in brain, from being inhibited by nerve agent (Polak and Cohen, 1969). Blood serves as a transporting medium for nerve agents and binding a portion of them by plasma CarbE decreases the amount of toxicant reaching the vital centers in the brain. This depot can be reactivated by DAM and MINA (Polak and Cohen, 1969, 1970a,b). The fact that the activity of these enzymes can be spontaneously reactivated explains the mentioned resistance of experimental animals to repeated administration of nerve agents (Stojiljković et al., 2004).

The scavenging role of CarbE is of particular importance in rodent species with large plasma CarbE activity (Myers, 1952; Aldridge, 1953; Goutier, 1956; Augustinsson, 1959; Christen and Cohen, 1969; Polak and Cohen, 1970a; Jokanović, 2001, 2015). Rat plasma is especially rich with CarbE; it was reported that the concentration of catalytic centers in rat plasma was as high as 2.8−3.2 μmol/L. Guinea pig plasma trails the rat's one, with a corresponding concentration of 0.5−0.6 μmol/L. All these active centers bind and detoxify the molecules of nerve agents (Christen and Cohen, 1969; Christen et al., 1969; Cohen et al., 1971). It was also shown that there were significant variations both in the plasma CarbE activities and in their sensitivity to inhibition with CBDP

even within the same species, that is, among the animal strains (Clement and Erhardt, 1990).

71.6 Toxicity of nerve agents and carboxylesterase

There are many proofs that a positive correlation exists between plasma CarbE activity and the LD_{50} of various OPCs and nerve agents in rodents. This means that the higher the plasma CarbE activity, the higher the LD_{50} (and the lower the toxicity) of nerve agents. Since developing young rats (i.e., until the age of 31 days) gain with time the CarbE activity, the LD_{50} of soman also increases and its toxicity proportionally decreases, with the difference between rats aged 31 days and those aged 5 days being six- to sevenfold. At the same time, ChE activity in tissues of developing rats remained fairly constant (Sterri et al., 1985a). Moreover, as a further proof of the concept, when rats aged 14 days (i.e., those ones with low CarbE activity) were treated i.v. with porcine hepatic CarbE, the resulting toxicity of soman was significantly reduced. In fact, only one of 10 animals died following inhalation exposure to soman, provided they received prophylactically a 60,000 molecular weight fraction of porcine liver CarbE (Fonnum et al., 1985). Maxwell et al. (1988) showed that in rats aged 3, 60, 90, and 120 days i.m. soman LD_{50} changes in a way that the corresponding LD_{50} values are 87, 117, 68, and 59 μg/kg, respectively, which correlates in a linear manner with the plasma CarbE activities of 3.6, 4.2, 2.5, and 1.0 μmol/L.

71.7 Carboxylesterase inhibitors

Two main CarbE inhibitors used in CarbE research are 3-*ortho*-cresyl phosphate (TOCP) (Mendel and Myers, 1953) and its metabolite 2-[*O*-cresyl]-4H-1,2,3-benzodioxa-phosphorin-2-oxide (CBDP) (Casida et al., 1961). Since TOCP needs to be activated, that is, biotransformed in liver to three metabolites, with CBDP as quantitatively the most important one, it has to be administered 24 h before poisoning with a nerve agent, with a wide dosage range of 3.5−250 mg/kg (Fonnum and Sterri, 1981; Jokanović, 1989), while the pretreatment interval for the active metabolite CBDP 35−50 mg/kg is 60−120 min (McKay et al., 1971; Bošković, 1979).

Pretreatment of rats and guinea pigs with TOCP resulted in a decrease in the value of soman LD_{50} to one-third of its control value (Fonnum and Sterri, 1981; Sterri, 1981; Sterri et al., 1981). Pretreatment with CBDP was even more effective, since it resulted in an 18-fold decrease of soman LD_{50} in mice (McKay et al., 1971; Bošković, 1979). Sarin LD_{50} was reduced by TOCP pretreatment in rats to one-fifth of the control value (Myers, 1959; Polak and Cohen, 1969). It was also discovered that inhibition of CarbE with CBDP leads to similar LD_{50} values of soman in mice, rats, and guinea pigs (Maxwell et al., 1988; Maxwell and Brecht, 2001).

Jokanović (1989) used two very different doses of TOCP for pretreatment of rats poisoned on the next day with either tabun, sarin, or soman—3.5 mg/kg that inhibited plasma CarbE by 90% and liver CarbE by 23% and 250 mg/kg that inhibited the enzyme in plasma by 100% and by 85% in liver. Relative potentiation of toxicities of tabun, sarin, and soman exerted by these two doses of TOCP were 1.1 and 1.4, 2.6 and 2.8, and 2.6 and 7.1, respectively, after s.c. injection of nerve agents, while the corresponding relative potentiations of their toxicity after i.p. administration were 1.6 and 2.2, 2.7 and 3.4, and 2.2 and 3.6, respectively. It is obvious that CarbEs have the greatest effect on soman, as the nerve agent most difficult to treat. The reason why the large TOCP dose was accompanied with twice as low impressive augmentation of soman toxicity after i.p. than after s.c. administration is the activity of liver somanases; namely only 27% of soman injected s.c. passes through the liver (Gaines et al., 1966), while all the i.p. administered soman has to pass this organ and become subject to degradation by its somanases (Bošković et al., 1988).

Jimmerson et al. (1989) pretreated rats with either of two doses of CBDP (1 and 16 mg/kg), which resulted in six- and eightfold decreases in soman LD_{50}, respectively. At the same time, the smaller dose of CBDP decreased the ED_{50} values for brain ChE inhibition and for the occurrence of soman intoxication by 10- and 7-fold, respectively. This is additional proof of the protective role of CarbE in rats, where under the conditions of their inhibition, soman attacks and inhibits ChE and AChE in the brain.

Various species have high (mice, rats), medium (guinea pigs), minor (marmosets), or zero (rhesus monkeys and humans) concentrations of plasma CarbE, respectively (Myers, 1952; Aldridge, 1953; Christen et al., 1969; Christen and Cohen, 1969; Cohen et al., 1971). This notion can afford an explanation why these species have so different LD_{50} values of soman (Dirnhuber et al., 1979; Sterri et al., 1980, 1981, 1985a).

A concept developed by Sterri and Fonnum (1989) is based on the assumption that the AChE pool in vital organs, such as brain, is relatively small and similar in several species, while the pool of plasma and liver CarbE depends on the species and may be very large (e.g., in mice) or nonexistent (e.g., in rhesus monkeys and humans) (Fig. 71.2).

This schematic presentation lists all the factors important for survival after intoxications with nerve agent: (1) AChE, (2) plasma CarbE, (3) carbamate prophylaxis, and (4) postexposure antidotes. In the case of species without plasma CarbE activity, such as humans, the

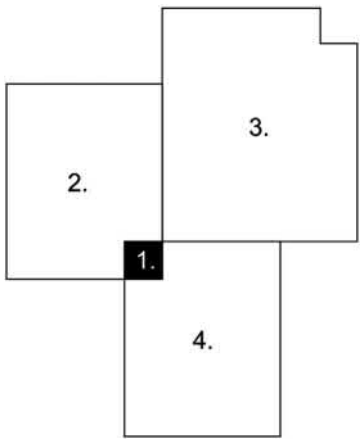

FIGURE 71.2 Visual representation of soman LD_{50} in rats as proportionally influenced by (1) target AChE, (2) plasma CarbE, (3) carbamate prophylaxis, and (4) other therapeutic regimen (at rat plasma CarbE concentration). The concentration of target AChE (1) or its enhancement by carbamate prophylaxis (3) is constant among the species, whereas the concentration of plasma CarbE (2) varies. The representation is based on the CarbE concept outlined by Sterri and Fonnum (1989) and Sterri (1989). LD_{50} decreases (and toxicity increases) when plasma CarbE (2) is decreased or absent.

segment (2) is nonexistent. This concept can be applied to the so-called G-agents tabun, sarin, and soman, while it is not applicable to VX, an OPC that does not bind to CarbE, but almost exclusively to AChE and ChE (Maxwell, 1992).

71.8 Carboxylesterase and prophylactic/therapeutic interventions

The area of segment (3) denoting protection offered by pyridostigmine prophylaxis is 30-fold larger than the area symbolizing the target AChE activity. As a consequence, the protective ratio (PR) in species that have a large pool of plasma CarbE (e.g., mice, rats, guinea pigs) can be calculated as the ration between (1) + (2) + (3) (i.e., LD_{50} in pyridostigmine-protected animals) and the same, but without carbamate prophylaxis (1) + (2). Since (2) is large in these species, the obtained PR is low. On the contrary, in rhesus monkeys the field (2) does not exist and the PR is calculated as the ratio between (1) + (3) and (1), which is around 30. This is why a similar level of pyridostigmine protection is to be expected in humans too, since they, like rhesus monkeys, lack plasma CarbE activity. Indeed, pyridostigmine prophylaxis along with atropine assured PRs against soman of 15 and 28 in marmosets, a species with low plasma CarbE activity and rhesus monkeys, a species completely without it, respectively (Dirnhuber et al., 1979).

The same applies to postexposure therapy, with or without carbamate protection—(1) + (2) + (3) + (4) or (1) + (2) + (4), respectively. In both cases the PRs are relatively modest in species with a large plasma CarbE pool and high in those with low (marmosets) or absent plasma CarbE activity (rhesus monkeys, humans).

71.9 Stoichiometric and catalytic scavengers of organophosphorus compounds

Indeed, it was later shown that the PRs obtained by prophylactic and/or postexposure antidotes in various species are similar in rats, guinea pigs, rabbits, and nonhuman primates, but only when their plasma CarbEs were previously inhibited by CBDP (Maxwell and Brecht, 1991; Maxwell et al., 1993). All these findings have important implications in further development of antidotal regimens against nerve agents in humans, since only the effects obtained in species with a naturally low level of CarbE or in those where the CarbE activity was rendered low by TOCP- or CBDP-induced pretreatment can be directly extrapolated to humans. A good example is use in such experiments of CarbE knockout mice (Duysen et al., 2012; Marrero-Rosado et al., 2018).

The idea of sequestration of nerve agents by an enzyme originated from the notion that CarbE, and especially that in plasma, can bind OPCs. For these purposes intravascular prophylaxis with CarbE, AChE, and BuChE of various origins became a new and promising field of research (Fonnum et al., 1985; Wolfe et al., 1987; Raveh et al., 1989, 1997; Broomfield et al., 1991; Maxwell et al., 1991, 1992; Doctor et al., 1993). In all these experiments B-esterases were used as stoichiometric scavengers, meaning that one molecule of an esterase neutralizes one molecule of an OPC. This approach has its limitations, such as the need for administration of large quantities and volumes of these esterases. In addition, in some experiments on wild-type human CarbE (hCE1) a spontaneous reactivation of the enzyme could be obtained only after adding sarin, but not when soman or cyclosarin were the inhibitors (Hemmert et al., 2010).

For these reason, A-esterases that decompose molecules of OPCs without being inhibited themselves—catalytic scavengers—are being intently investigated. Human paraoxonase-1 (PON-1) is certainly one of them, although it possesses only a moderate affinity to bind OPCs and has a slow turnover rate, meaning that it takes some time before this enzyme becomes ready to bind another OPC molecule. It is therefore not likely that the prophylactic use of native human PON-1 can result in significant protection against nerve agents (Lenz et al., 2007). For this reason, further efforts are being undertaken in order to develop mutants of human PON-1 (Yeung et al., 2004; Aggarwal et al., 2016). Although the results of the initial

efficacy experiments are promising (Worek et al., 2014), their immunogenicity and plasma half-lives in humans have yet to be determined (Lenz et al., 2007).

There are also reports on engineering a slightly modified molecule of human CarbE1, where histidine was introduced instead of valine (V146), and glutamate instead of leucine (L363). Charges of valine and glutamate positioned on the opposite sites of the CarbE active center direct a water molecule toward the hydroxyl group of serine and thus alleviate CarbE reactivation. The resulting molecule retained the same affinity for binding the OPCs, with the spontaneous reactivation time being much shorter (Hemmert et al., 2011).

71.10 Concluding remarks and future directions

CarbE proved to be very important for species-dependent detoxification of nerve agents tabun, sarin, and especially soman. Experimental models for their induction with barbiturates and inhibition with TOCP or its metabolite CBDP enable direct extrapolation of the protection experiments from rodent species (with abundant CarbE plasma activity) to humans (with absence thereof). CarbEs can be used as effective stoichiometric scavengers against nerve agents, but with long spontaneous reactivation times. Further research is warranted to investigate the possibility to develop a modified CarbE molecule with unchanged affinity to bind nerve agents, but with a significantly shorter turnover time.

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Chapter 72

Catalytic bioscavengers: the second generation of bioscavenger-based medical countermeasures

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Abbreviations

AChE	acetylcholinesterase
BChE	butyrylcholinesterase
CaE	carboxylesterase
ChE	cholinesterase
CRISPR	clustered regularly interspaced short palindromic repeats
CVX	Chinese VX
DFP	diisopropylfluorophosphate
DFPase	diisopropylfluorophosphate hydrolase
GF	cyclosarin, cyclohexylsarin
GST	glutathione *S*-transferase
HPBP	human phosphate binding protein
MD	molecular dynamics
NA	nerve agent
NTE	neuropathy target esterase
OP	organophosphorus compound
OPAA	organophosphoric acid anhydrolase
OPAH	organophosphorus acid anhydride hydrolase
OPH	OPase organophosphate hydrolase
PAF-AH	platelet-activating factor acetylhydrolase
PI	penta-coordinated intermediate
PLL	phosphotriesterase-like lactonase
PON-1	paraoxonase 1
PROL	prolidase
PTE	phosphotriesterase
QM/MM	quantum mechanics/molecular mechanics
SMP	senescence marker protein
TCP	tricresyl phosphate
TMPP	trimethylolpropane phosphate
VX	ethyl ({2-[bis(propan-2-yl)amino]ethyl}sulfanyl)(methyl) phosphinate
VR	Russian VX

72.1 Introduction

Organophosphate thio/oxo-esters (OPs) were discovered in the middle of the 19th century. They are mostly pesticides (parathion, malathion, chlorpyriphos, dichlorvos), and some of them have been used as drugs or prodrugs (echothiophate, metrifonate, cyclophosphamide), flame retardants, and antiwear agents (tricresyl phosphate, TCP). Other OPs are potent chemical warfare agents (G agents: tabun, sarin, soman, cylclohexyl-sarin, and V agents: VX, VR, CVX). The fourth-generation NAs, called "novichoks" (A-230, A-232, A-234), are phosphoramidates supposedly 10 times more toxic than V agents (Tucker, 2006; Mirzayanov, 2009; Hosseini et al., 2016; Carlsen, 2018; Nepovimova and Kuca, 2018; Franca et al., 2019; Kuca and Nepovimova, 2019). Highly toxic OPs may be formed by cytochrome P450-mediated metabolic oxidation of thiono-phosphoesters, for example, paraoxon from the parent compound parathion (Foxenberg et al., 2007), or 2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP or cresyl-saligenin phosphate, CSP) by cyclization of tri-*ortho*-cresyl phosphate (TOCP), a TCP isomer (Eto et al., 1962). High-temperature pyrolysis of synthetic oils may lead to toxic OPs, such as trimethylolpropane phosphate (TMPP) (Masson, 2012). Most of these compounds are potent irreversible inhibitors of cholinesterases (ChEs) (Fig. 72.1) (Costa, 2006), and of many other serine hydrolases. However, TMPP is not an inhibitor of ChEs; it is a GABA antagonist, so toxic that it has been called "the poor man's nerve agent."

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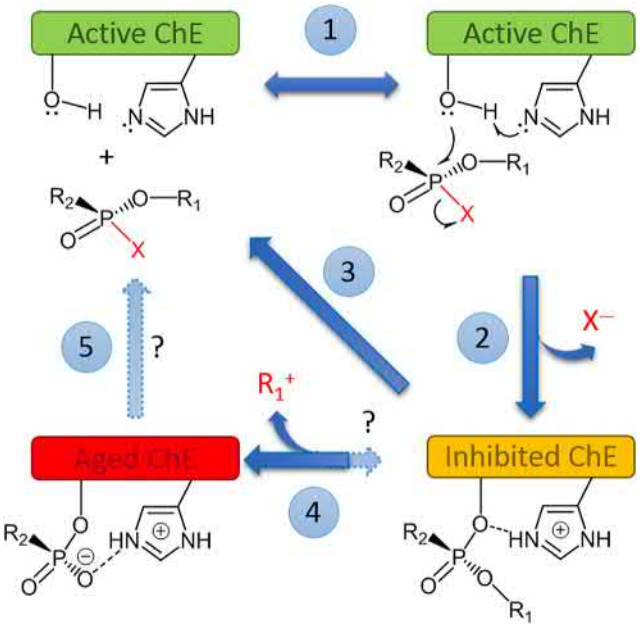

FIGURE 72.1 Mechanism of inhibition of cholinesterases by OPs. After formation of reversible complex between ChE and OP (step 1), the active serine (esteratic site, E-OH) is phosphylated; the reaction leads to release of leaving group X (step 2). The phosphylated enzyme can be reactivated by nucleophilic agents, such as quaternary oximes [2-PAM (contrathion or pralidoxime), TMB-4, MMB-4 (methoxime), Obidoxime (toxogonin), HI-6, Karboxim (carboxim)], used as antidotes in emergency treatment of OP poisoning (Lundy et al., 2006; Worek et al., 2007; Worek and Thiermann, 2013) (reaction 3); water is a too weak nucleophile for fast spontaneous reactivation of phosphylated ChE. The phosphyl–ChE conjugate may undergo a spontaneous dealkylation through alkyl-oxygen bond scission ("aging") (Kovach, 2004; Masson et al., 2010), resulting in irreversibly inactivated ("aged") enzyme (step 4). The dealkylation reaction can be very fast ($t_{1/2} = 3$ min at 37°C for human AChE phosphonylated by soman). Drug-mediated reactivation to E-OH through realkylation (R_1) of aged ChE (reverse reaction 4 and subsequent reaction 3, called "resurrection" or "resuscitation") was recently demonstrated to be possible (Zhuang et al., 2018). Direct displacement of the aged adduct (reaction 5) is still thought to be impossible.

There are two types of ChEs, acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC.3.1.1.8). AChE plays a major role in the cholinergic system terminating the action of the neurotransmitter acetylcholine. Inhibition of AChE leads to accumulation of acetylcholine in synapses, and blockade of cholinergic transmissions in the peripheral and central nervous systems. Inhibition of AChEs is the main cause of acute toxicity of OPs (Maxwell et al., 2006). Irreversible inhibition of other enzymes, including various serine hydrolases plays a role in subacute toxicity and noncholinergic toxicity of OPs (Casida and Quistad, 2004, 2005). Phosphylation of serine, tyrosine, lysine, and other residues in numerous proteins is also involved in chronic toxicity of OPs (Onder et al., 2018; Schopfer and Lockridge, 2019).

Significant progress has been made in the past 25 years in emergency treatments of acute poisoning and management of poisoned casualties (Eyer et al., 2007; Thiermann et al., 2007; Wetherell et al., 2007; Eddleston et al., 2008; Moshiri et al., 2012; Masson, 2016). However, classical pharmacological approaches are reaching their optimum limit. Recent attacks in Syria, where nerve agents (NAs) were employed, revealed the limitations of current medical treatments (Dolgin, 2013). In addition, due to accumulation of OP in depot sites and subsequent slow release from these stocks, blood and tissue ChEs may remain inhibited for long periods of time. Therefore, persistence of certain OPs in the body after initial exposure complicates treatments. This is particularly well documented for severe intoxications by parathion (Willems et al., 1993).

Endogenous enzymes are involved in natural defenses against OP toxicity. The presence of detoxifying enzymes in skin contributes to reduce the OP dose that penetrates the body (Schallreuter et al., 2007). Numerous secondary targets of OPs present in various tissues are detoxifying enzymes and they play a role in the natural defenses against OPs (Wang et al., 1998; Nomura et al., 2005, 2008). The presence of endogenous OP-hydrolyzing enzymes has been regarded as the first line of defense against OP poisoning (Satvik Iyengar et al., 2015).

Liver enzymes play an essential role in detoxification. In particular, oxidases, such as glutathione *S*-transferases (GSTs), play a role in degradation of alkyl/aryl chains (Casida and Durkin, 2013). However, as mentioned above, liver cytochrome P450 enzymes activate phosphorothioates and lead to oxon forms much more toxic than parent compounds, but cytochromes P450 also dearylate aryl-containing OPs and participate in detoxification of OPs (Furlong, 2007; Ellison et al., 2012). Natural blood bioscavengers significantly contribute to reduce the amount of OP molecules reaching physiological targets. It has been shown that animal species in which the concentration in paraoxonase-1 (PON-1; EC 3.1.8.1) or in carboxylesterase (CaE; EC 3.1.1.1) is high are relatively resistant to OPs (Kaliste-Korhonen et al., 1996). Conversely, *knockout* mice for PON1 are very sensitive to OPs (Shih et al., 1998). Albumin displays a low esterase activity, and slowly reacts with carbamyl- and phosphoryl-esters. However, its concentration in blood and lymph is so high (≈ 0.6 mM) that it likely plays a role in detoxification of carbaryl at toxicologically relevant concentrations (Sogorb et al., 2007). Thus, plasma albumin could also play a role in the detoxification of certain OPs (Tarhoni et al., 2007; Li et al., 2008).

Toxicity of OPs can be countered by reducing skin absorption and lowering OP concentration in the blood compartment, thus preventing the transfer of OP molecules toward cholinergic synapses and other biological

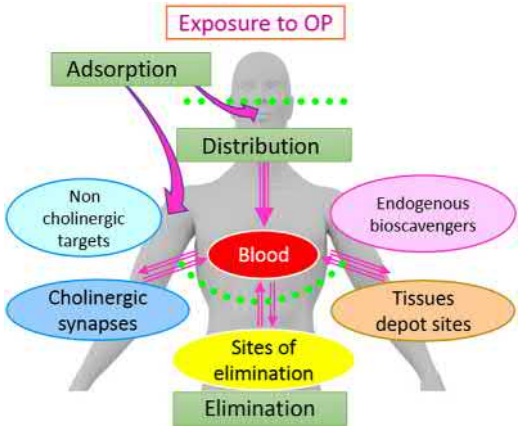

FIGURE 72.2 Biological fate of organophosphorus compounds in human.

Routes of penetration of OPs are absorption through the skin, eyes, and/or respiratory tract (NAs, pesticides), or ingestion (self-poisoning). OP molecules distribute from the blood compartment into tissues, including depot sites, biophase (cholinergic synapses and secondary targets) and sites of elimination (liver and kidneys). Cholinesterases are the main biological targets (acute toxicity); reaction with secondary targets (carboxylesterases, serine-amidases, peptidases, and other proteins) may be responsible for noncholinergic sublethal effects of OPs and chronic toxicity at low-dose exposure (Costa, 2006; Bigley and Raushel, 2013).

targets (Fig. 72.2). Neutralization of toxicant molecules can be achieved by using stoichiometric traps or catalysts acting on exposed surfaces (e.g., active topical skin protectants, TSPs) or in the bloodstream (bioscavengers). The concept of bioscavengers and developments of this approach in prophylaxis and postexposure treatment of OP poisoning have been exposed in several reviews (Masson and Rochu, 2009; Masson and Lockridge, 2010; Nachon et al., 2013; Masson and Nachon, 2017).

72.2 Stoichiometric scavengers

The first molecules that were studied for the purpose of making stoichiometric scavengers were cyclodextrins (Desire and Saint-Andre, 1986), neutralizing antibodies (Glikson et al., 1992), and activated charcoal. Though the interest in charcoal is much debated, hemodialysis on a charcoal cartridge was successfully used on a Tokyo casualty who was resistant to the classical treatment of sarin poisoning (Yokoyama et al., 1995). Transfusion of human plasma has been used for treating OP poisoning. The effects of fresh frozen plasma (plasmapheresis) on cholinesterase levels and outcomes in OP-poisoned patients have been evaluated (Güven et al., 2004; Qiu et al., 2011). An in vitro study also suggests that plasma (freshly prepared and fresh frozen plasma) therapy may be an effective adjunctive treatment method against G agents (Wille et al., 2014). Plasma BChE, and possibly other abundant OP-scavenging proteins in plasma (e.g., albumin, PON-1), may have contributed to these results. At this point, it must be noted that unlike plasma of most model animals, human plasma does not contain carboxylesterases (Li et al., 2005; Napon et al., 2018).

At the end of the 1980s, research on scavengers mostly focused on enzymes that specifically react with OPs. Cholinesterases (Wolfe et al., 1987) and carboxylesterases (Redinbo and Potter, 2005; Fleming et al., 2007; Hemmert et al., 2010) have been proposed as stoichiometric scavengers. Human BChE has proved to be an effective stoichiometric bioscavenger for pre- and postexposure treatment of NA and OP pesticide poisoning (Allon et al., 1998; Doctor and Saxena, 2005; Lenz et al., 2007; Saxena et al., 2008a,b; Mumford et al., 2013; Rosenberg et al., 2013, 2014; Rice et al., 2016; Reed et al., 2017). Human plasma-derived BChE was granted Investigational New Drug (IND) status by the FDA in 2006 for protection against nerve agents (Lenz et al., 2007). Clinical trials on volunteers (phase I) were then performed. However, enzymatic stoichiometric neutralization of several OP LD_{50} needs administration of huge amounts of enzyme, about 3 mg/kg of highly purified plasma BChE (Ashani and Pistinner, 2004), that is, 200−300 mg for humans against $2 \times LD_{50}$ of soman. This enzyme must be free of contaminants, for example, coagulation factors, bacterial toxins, viruses, and bacteria. Large-scale production of enzymes, in particular human plasma BChE, under GMP conditions at a reasonable cost has been the subject of intense research in the past 10 years. However, although several thousand tons of outdated human plasma or Cohn fraction IV are available in the United States for preparing the enzyme, 1 L of human plasma provides less than 1 mg of GMP BChE tetramer.

Several industrial GMP processes have been proposed for mass production of human BChE. The first is purification of the natural enzyme from human plasma (Cohn fraction IV). This process was initially developed by Saxena et al. (2008a,b) and Baxter Healthcare Corporation (www.baxter.com). The second method was developed by Nexia (www.nexiabiotech.com), and uses the recombinant human enzyme produced in milk of transgenic goats. Several grams of enzyme can be secreted in 1 L of milk. This enzyme has been named Protexia. Pharmatheme (www.pharmathene.com), in 2005, developed Protexia PEGylated derivatives of this enzyme (Huang et al., 2007) and fusion proteins (Huang et al., 2008). However, production of recombinant human BChE in milk of transgenic mammals has been discontinued due to high cost and pharmacokinetics problems. Therefore, Pharmatheme and other companies are working on large-scale production of recombinant human BChE expressed in human cells or in CHO cells (Rosenberg et al., 2010). Production of tetrameric human BChE in transgenic tobacco (*Nicotiana benthamiana*) has

also been made possible (Geyer et al., 2010; Larrimore et al., 2013; Alkanaimsh et al., 2016). PlantForm in Canada (www.plantformcorp.com) has been engaged in the development of recombinant human BChE in tobacco that mimics the stability and pharmacokinetics of plasma-derived enzyme. In Russia, expression of human BChE in horseradish has been undertaken with mixed results (Martirossian et al., unpublished). After years of failure, functional expression in bacteria of both human AChE (Goldenzweig et al., 2016) and human BChE (Brazzolotto et al., 2017) is now possible. Even functional expression of thermally stable human BChE dimer in *Escherichia coli* has recently been possible (Cai et al., 2019). However, this approach implies introducing a large number of mutations in the protein structure. This enzyme redesign may impair further mutagenesis of the enzyme, for example, for making OP-degrading ChEs.

The recent introduction of Hupresin for affinity chromatography of recombinant or human plasma has made it possible to speed up purification of cell cultures, Cohn fraction IV, or transgenic plant extracts (Lockridge et al., 2018; Alkanaimsh et al., 2019; Schopfer et al., 2019), even at a preindustrial scale (Schopfer et al., 2019).

Certain secondary targets of OPs are potential bioscavengers. In particular, owing to the high number of amino acid residues in human serum albumin that covalently bind OP molecules (five tyrosines and two serines) (Ding et al., 2008), it may be hypothesized that reactivity of these residues could be enhanced by genetic engineering and/or upon specific chemical modification. However, direct tyrosine nitration (to lower the pK_a of tyrosine) did not lead to the expected improvement in reactivity, possibly because of steric hindrance (Masson et al., unpublished). Engineered albumins could lead to a new generation of stoichiometric bioscavengers. However, conversion of albumin into a catalytic bioscavenger would need to increase its catalytic efficiency by several orders of magnitude, a challenge that seems to be unrealistic (Li et al., 2008).

Low-molecular-weight stoichiometric scavengers could be an economic alternative to enzyme-based stoichiometric scavengers. Several serine- and tyrosine-containing hexapeptides from a random library of peptides have been selected because they form a phosphoester bond with a fluorescent analog of sarin (Landry and Deng, 2008; Zhu et al., 2008). However, the reactivity of these molecules is not yet sufficient. Finally, a way to increase the endogenous stoichiometric bioscavenger capacity could be to enhance the endogenous expression of BChE and AChE. This could be achieved by administration of proline-rich polypeptides derived from ChE collagenic tail peptide (ColQ, PRAD) or peptides derived from RAPH-1 or transmembrane anchor (PRIMA) that promote folding, tetrameric assembly, and exportation of the enzymes from cells (Rotundo, 2011). The recent resolution of the tetrameric structure of natural human BChE (Leung et al., 2018; Boyko et al., 2019) and information about the structural organization of the C_5 variant of hBChE, composed of the BChE tetramer linked to a polyproline protein of 50 kDa (Schopfer et al., 2017) are expected to help to make more stable ChE-based bioscavengers with longer residence time in the bloodstream. Human umbilical cord perivascular cells secrete a BChE tetrameric form related to the C_5 variant. Cultures of these cells could be developed to produce wild-type or engineered BChE (Braid et al., 2019).

72.3 Pseudocatalytic bioscavengers

Since the main limitation of the stoichiometric bioscavenger approach is the cost of the huge dose of enzyme to be administered for challenging the OP molecules, a way to circumvent this problem is to in vivo reactivate the administered enzyme, turning the stoichiometric bioscavenger into a pseudocatalytic bioscavenger.

Certain ChE mutants sensitive to OPs do not "age" after phosphylation, they are fully reactivatable (cf. Fig. 72.1, reaction 3). Such ChE mutants, for example, the human AChE mutant Y337A/F338A (Cochran et al., 2011) when associated with oximes (e.g., 2-PAM, HI-6), act as pseudocatalysts in displacing the OP moiety bound to the enzyme. These enzyme−reactivator coupled systems could lead to a new family of pseudocatalytic bioscavengers (Kovarik et al., 2007, 2008; Taylor et al., 2007; Mazor et al., 2008). Study with wild-type human BChE showed that the geometry of oxime function access to the phosphorus atom of conjugate is an important criterion for fast reactivation (Semenov et al., 2020). Several libraries of positively charged and uncharged molecules have already been made by in silico design or click chemistry and tested with success in vitro against human ChE inhibited by paraoxon, tabun, soman, and VX (Kovarik et al., 2013; Radic et al., 2013; Renou et al., 2013; Katalinic et al., 2016, 2018).

The success of this approach in vivo requires implementation of new oximes, displaying higher affinity for phosphylated ChEs (lower K_D), higher reactivation constant (k_r), and a long circulation time in the bloodstream. However, for reactions under optimal conditions, the pharmacokinetic profiles of enzymes and reactivators must be similar. Though enzymes can be chemically modified for long residence times in the bloodstream, pharmacokinetics of oximes is in general fast. To circumvent this problem, oximes can be either encapsulated into nanocontainers for prolonged release (Pashirova et al., 2018) or both enzyme and encapsulated oximes incorporated into

circulating nanoreactors where coupled reactions of bioscavenger phosphylation and oxime-mediated reactivation take place (Masson et al., unpublished).

72.4 Catalytic scavengers

Catalytic scavengers are second-generation bioscavengers. They are enzymes or artificial catalysts capable of degrading OPs with a turnover. These catalysts detoxify OPs by hydrolyzing the phosphoester [organophosphorus acid anhydride hydrolase (OAAH) activity also called "phosphotriesterase" (PTE) activity, OP hydrolase (OPH, OPase) activity]. Other catalytic bioscavengers lead to less toxic compounds by degrading their alkyl/aryl chains through oxidation. Several recent reviews deal with this second generation of bioscavengers (Masson and Lushchekina, 2016; Worek et al., 2016; Masson and Nachon, 2017; Goldsmith and Ashani, 2018). In particular, the last one discusses the recent in vivo outstanding protective results obtained with evolved PTEs.

The catalytic bioscavenger concept is based on the idea of continuously trapping and degrading OPs in the bloodstream before OP molecules reach their central and peripheral neuronal and neuromuscular targets. Then, prophylactic injection of enzymes capable of hydrolyzing OP quickly (alone or in association with current prophylactic countermeasures, i.e., pyridostigmine bromide, PANPAL, and other reversible ChE inhibitors and anticholinergic drugs) (Masson, 2016), would allow first responders, firemen, explosive ordnance disposal technicians, and medical personnel, to operate safely in contaminated environments on contaminated casualties. Intravenous or intramuscular administration of bioscavengers to chemical casualties is expected to greatly improve the efficacy of implemented pharmacological countermeasures (Ashani et al., 1998; Saxena et al., 2006).

These enzymes could be also used for protection of skin (Fischer et al., 2005), and for decontamination of skin, mucosa, and open wounds (LeJeune and Russell, 1999; Gill and Ballesteros, 2000). Genetically engineered bacteria producing OPHs could be introduced in water effluents of decontamination units that could purify contaminated water before recycling or washing up in the environment (Chen and Mulchandani, 1998).

Research on catalytic antibodies has made some progress (Jovic et al., 2005; Smirnov et al., 2013). In particular, resolution of the 3D structure of a "reactibody" fragment with an OP (Smirnov et al., 2011) has opened the possibility of rational design of more active catalytic antibodies (Kurkova et al., 2012). However, the turnover rate of catalytic antibodies is still extremely slow, and antibody specificity remains too narrow for practical interest as medical countermeasures.

Research on artificial enzymes acting as catalytic scavengers, for example, functionalized β-cyclodextrins, is promising. Cyclodextrin derivatives bearing a nucleophilic group such as isodozobenzoic acid or an oxime were made (Ramaseshan et al., 2006; Estour et al., 2013; Kalakuntla et al., 2013; Bierwisch et al., 2014; Letort et al., 2016; Sambrook et al., 2017). These compounds display very interesting catalytic properties against certain NAs, in particular cyclosarin (Muller et al., 2013). However, engineering (computer design and/or directed evolution) of enzymes capable of degrading OPs remains the most promising short-term research field.

72.5 Requirements

The general requirements for the use of enzyme-degrading OPs as medical countermeasures against OP poisoning are as follows. These enzymes must have a large activity spectra, and ideally, enantioselectivity for toxic stereoisomers. Their mass production under GMP conditions must be realizable at a reasonable cost. Long-term storage without activity loss (in solution, lyophilized, or adsorbed/bound on a matrix) must be possible under field conditions. Conformational stability can be optimized by chemical modification or addition of stabilizers likes polyols. Otherwise, thermostable phosphotriesterases from hyperthermophilic bacteria (Merone et al., 2005; Elias et al., 2008) expressed in *E. coli* or mutated/evolved highly stable enzymes from mesophilic bacteria are promising alternatives.

Other conditions depend on the method of administration, delivery system, or galenic formulation of these enzymes. Enzymes can be injected intravenously or intramuscularly. Other routes have been considered such as the intranasal method against aerosolized or gaseous NAs. For instance, pretreatment of macaques with aerosolized forms of BChE have been shown to provide long-term protection against aerosolized paraoxon (Rosenberg et al., 2013, 2019). For parenteral administration, the toxicant concentration in blood has to be considered. Even in the most severe case of poisoning, this concentration, [OP], is always very low. For example, the sarin concentrations in serum of casualties after Matsumoto and Tokyo chemical attacks have been estimated between 1.5 and 30 nM, 14 h postexposure (Polhuijs et al., 1997). Therefore, the [OP] concentration in plasma is always well below the K_m of the enzyme for OP substrates. This determines first-order kinetics for hydrolysis of OP in blood (Masson et al., 1998, 2008) as described by Eq. (72.1):

$$v = k_{cat}/K_m \cdot [E] \cdot [OP] \qquad (72.1)$$

In Eq. (72.1), the product of the bimolecular rate constant (k_{cat}/K_m) and the enzyme active site concentration ([E]) is the first-order rate constant. The amount of

enzyme to be injected for degradation of toxic molecules in a very short time depends on the enzyme efficiency, that is, k_{cat}/K_m. The higher the catalytic efficiency, the lower the enzyme dose to be administered. The enzyme concentration needed to drop the OP concentration to a nontoxic concentration in time t is:

$$[E] = \frac{X}{k_{cat}/K_m \cdot t} \tag{72.2}$$

X is the factor by which [OP] is reduced ($X = \mathrm{Ln}[OP]_0/[OP]_t$) (Masson et al., 2008). In this equation, it is assumed that the stability and pharmacokinetics of the administered enzyme have been optimized, and that the enzyme concentration, [E], does not decrease during the time-course of the reaction with OP. The efficiency and stereospecificity of a given enzyme can be increased by several orders of magnitude by mutagenesis or chemical engineering (Griffiths and Tawfik, 2003; Hill et al., 2003; Gupta et al., 2011; Tsai et al., 2012; Goldsmith et al., 2017; Goldsmith and Ashani, 2018). Engineering strategies to increase k_{cat}/K_m have been theorized (Goldsmith and Tawfik, 2017).

The second constraint is to maintain the bioscavenger concentration, [E], in the bloodstream as high as possible for a long time. [E] is controlled by the enzyme pharmacokinetics/pharmacodynamics and/or the frequency of repeated administrations (sustained pharmacokinetics). Increasing the size of the enzyme by polymerization, conjugation to other proteins (e.g., albumin, antibody fragments) or to biodegradable polymers such as XTEN polypeptides (Podust et al., 2016), decreasing glycosylation microheterogeneity, and chemical modifications of solvent-exposed surface ("capping") improve the biological life of injected enzymes (Cohen et al., 2006). Fast clearance of glycoproteins is often due to glycosylation defects; fast clearance of nonglycosylated enzyme may result from their too small size (Jackson et al., 2010). Glycosylation defects can be corrected either by chemical modifications such as PEGylation (Chilukuri et al., 2005), polysialylation of purified expressed enzyme (Ilyushin et al., 2013), or by selection of an appropriate expression system (Chitlaru et al., 1998), including overexpression of an additional glycosylation enzyme that increases the sialylation content of the expressed glycoprotein (Schneider et al., 2014). Size defects can be corrected by conjugation to polyethylene glycol, dextran, other macromolecules, or fusion to albumin (Huang et al., 2008). Tetramerization of recombinant human BChE expressed in CHO cells was shown to considerably increase the residence time of the enzyme in mouse bloodstream (Terekhov et al., 2015). All modifications reduce renal clearance and increase plasma retention. Finally, like many detoxifying enzymes (Liu et al., 2015), nanoencapsulation of bioscavengers into nanocarriers ("nanoscavengers") may greatly increase their residence time in the bloodstream (Zhang et al., 2019) and suppress potential adverse effects such as immunoreactivity.

Administration of homologous enzymes does not induce an immunologic response following a second injection (Sun et al., 2009). On the other hand, immunotolerance of injected heterologous enzymes is a major issue. Bacterial enzymes and heterologous mammalian enzymes are not suitable for use in humans, but conjugation to dextran or polyethylene glycol (PEGylation) is often sufficient to reduce antigenicity and to slow down clearance following multiple injections (Novikov et al., 2010; Trovaslet-Leroy et al., 2011; Sun et al., 2013). Nanoencapsulation of nonhuman enzymes is another strategy to cheat the immune system and to increase residence time in the bloodstream (Liu et al., 2015; Zhang et al., 2019). However, enzyme-containing nanocontainers must be completely sealed to prevent leaks. This implies sophisticated design of decorated and cross-linked multilayer nanoparticles.

Therapeutic plasma exchange has proven to be effective in patients with severe intermediate syndrome resulting from severe poisoning by pesticide OPs (Yilmaz et al., 2013). Alternatively, extracorporeal removal of toxicants can by performed by hemodialysis (Borkan, 2002; de Pont, 2007; Monaghan and Acierno, 2011). Extracorporeal dialysis has been successfully implemented in a patient for blood decontamination after the Tokyo subway attack (Yokoyama et al., 1995). Moreover, it was postulated that charcoal hemoperfusion associated with infusion of lipid emulsions could be used for the treatment of severe OP pesticide poisoning (Zhou et al., 2010). Also, incorporation of OP-degrading enzymes in the medical dialysis system could greatly improve the efficiency of hemodialysis. Enzymes can be immobilized on dialysis cartridges (Klein and Langer, 1986). Accessibility of OP molecules to an enzyme active center must not be altered by the immobilization method or by matrix effects. The enzyme concentration per surface unit has to be maximized to reduce diffusion constraints. In that case, both the reactive surface of the matrix and k_{cat}/K_m would have to be as high as possible and the flow rate reduced to increase the efficiency of the reactor. First-order degradation kinetics takes place under the particular conditions of immobilized enzymes in a continuous-flow system. Immunocompatibility problems are theoretically suppressed, thus permitting the use of unmodified nonhuman enzymes. However, implementation of this approach needs blood derivation through peristaltic pump systems. The above-mentioned nanoreactor approach could be an alternative to extracorporeal immobilized enzyme-cartridges.

Lastly, in situ transient production of enzymes, if the need arises, will be possible by gene therapy in the future.

Promising results have been published using short-induction gene vectors (adenoviral systems) for human PON-1 (Cowan et al., 2001; Bradshaw et al., 2005; Fu et al., 2005; Miyoshi et al., 2007; Guns et al., 2008; Duysen et al., 2011), human AChE (Li et al., 2006), human BChE (Chilukuri et al., 2009; Parikh et al., 2011), mutated human BChE that displays high cocaine esterase (Gao et al., 2013), and human prolidase (Aleti et al., 2013).

Enzymes in skin and eye lotions, immobilized in foams and on tissues for skin and eye decontamination (Gordon et al., 2003), or in TSPs (Braue et al., 2002), act under conditions where local OP concentrations can be very high. In these cases, enzyme reaction order in [OP] tends to zero, so that the reaction rate is close to maximum velocity:

$$v \rightarrow V_{max} = k_{cat} \cdot [E] \qquad (72.3)$$

The enzyme efficiency depends on its concentration and its catalytic constant k_{cat}. Thus, for external use, enzyme preparations have to be highly concentrated with high molecular catalytic activity. Coimmobilization of different enzymes could be an easy way to extend the spectra of agents to be degraded. This should allow simultaneous detoxification of G and V agents, as well as other potential chemical warfare agents, pesticides and so-called "nontraditional nerve agents." Indeed, exposure to multiple agents has to be considered. In this view, it should be remembered that during the war between Iran and Iraq, tabun and other OPs were combined with mustard gas in some attacks (UN Reports, 1984, 1987). In asymmetric conflicts, eschatological and mafia-like terrorisms, the most extreme scenarios have to be taken into account.

72.6 Potential enzymes

72.6.1 Phosphotriesterases

72.6.1.1 Bacterial phosphotriesterases

Bacterial phosphotriesterases (PTEs; EC 3.1.8.1) detoxify OPs (Ghanem and Raushel, 2005; Theriot and Grunden, 2011). These enzymes have been isolated from numerous sources. Four enzyme families showing different folds or topologies can be described: TIM-barrel fold, β-lactamase fold, pita bread fold, and β-propeller fold (Bigley and Raushel, 2013). They are encoded by the organophosphate degradation (*opd*) gene found in species of *Brevundimonas diminuta* (formerly *Pseudomonas diminuta*), *Flavobacterium* sp., *Agrobacterium radiobacter* (Horne et al., 2002), and *Pseudomonas pseudoalcaligenes* (Gotthard et al., 2013), and genes similar to *opd* were also located in archaea (Merone et al., 2005). PTEs belong to a superfamily of amidohydrolases (Holm and Sander, 1997; Bigley and Raushel, 2013).

B. diminuta PTE is a 72 kDa dimeric bimetallic enzyme with Zn^{2+} involved in the catalytic process (Carletti et al., 2009). Substitution of the native Zn^{2+} ions in the active site with Mn, Co, Ni, or Cd ions results in almost full retention of catalytic activity. Following the first determination of the three-dimensional structure of *P. diminuta* PTE (Benning et al., 1994) (Fig. 72.3A), a

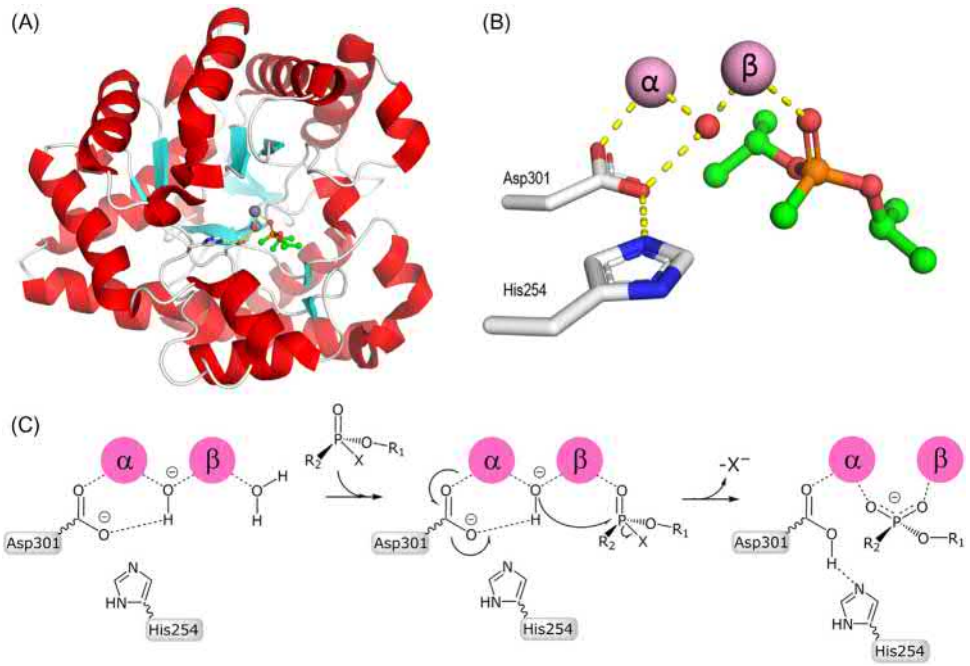

FIGURE 72.3 X-ray structure *Brevundimonas diminuta* PTE [PDB ID 1EZ2, (Benning et al., 2000)], (A) overall view and (B) close view on metal center with bound substrate analog diisopropyl methyl phosphonate. (C) Scheme of hydrolysis reaction described in Bigley and Raushel (2019).

series of crystal structures, kinetic, and spectroscopic experiments were described. Oxygen atom seen in X-ray structures, coupled with two metal cations (Fig. 72.3B), is thought to be in a hydroxyl form, because the structure is pH-dependent, protonation of the hydroxyl leads to loss of coupling (Samples et al., 2005). Nevertheless, the enzyme mechanism of bacterial PTEs is still debated and the functional roles of divalent metal cations and amino acids in the active center are not yet fully understood (Aubert et al., 2004; Jackson et al., 2006, 2008; Chen et al., 2007; Samples et al., 2007; Wong and Gao, 2007; Bigley and Raushel, 2013).

The mechanism proposed by Bigley and Raushel (Bigley and Raushel, 2013, 2019) suggests that OP hydrolysis by bacterial PTE occurs through direct attack of hydroxyl-group bridging divalent metal cations on P atom (Fig. 72.3C). As a result, formation of products is accompanied by inversion of phosphorus atom stereoconfiguration. Product is bound to cations in a bidentate manner. Surrounding residues have a supplementary role, accepting proton from the hydroxyl-group upon formation of negatively charged product. There are kinetic (Samples et al., 2005), crystallographic (Kim et al., 2008), EPR spectroscopy (Samples et al., 2007), NMR (Bigley et al., 2016), and computational (Wong and Gao, 2007; Zhang et al., 2009) supports for this mechanism.

These promiscuous enzymes are primarily lactonases, now called phosphotriesterase-like lactonases (PLL). The lactonase activity plays a role in bacterial communication (*quorum* sensing) (Dickschat, 2010). Virulence and formation of biofilms are regulated by concentration of lactones (*N*-acetyl-homoserine lactones) in the medium. Thus, the lactonase activity by hydrolyzing lactones acts as a *quorum* quencher, which in turn inhibits bacterial communication (Amara et al., 2011). The PTE activity of these enzymes is believed to have evolved from lactonases (Afriat et al., 2006; Elias et al., 2008; Afriat-Jurnou et al., 2012; Hiblot et al., 2012).

Whereas the catalytic efficiency of *B. diminuta* PTE for hydrolysis of paraoxon, the best substrate identified so far, is approaching the diffusion-controlled limit, it is slow against OP NAs (Table 72.1). Meanwhile, directed evolution of *B. diminuta* PTE showed that only three amino acid changes dramatically enhanced the catalytic efficiency for an analog of soman by about three orders of magnitude (Hill et al., 2003). Further studies combining rational design and directed evolution led to randomized libraries of mutants and selection of variants with greatly improved catalytic activity against S_p enantiomers of NA chromogenic analogs, including VX and VR analogs, and racemic real NAs (Table 72.1) (Tsai et al., 2010, 2012). This strategy has recently led to multiples variant with k_{cat}/K_m values up to four orders of magnitude higher than that of wild-type PTE against V agents (Bigley et al., 2019; Bigley and Raushel, 2019). Though a recent theoretical study suggests that enzymatic hydrolysis of novichok agents would be possible (Lyagin and Efremenko, 2019), it appears to be a very difficult challenge, owing to the presumed phosphoramidate structure of A-230, 232, and 234. There have been numerous studies highlighting the potential of this enzyme for decontamination or skin protection in addition to OP-detection (LeJeune and Russell, 1999; Gill and Ballesteros, 2000; Ghanem and Raushel, 2005; Letant et al., 2005; Karnati et al., 2007). Administration of PTE before or after OP exposure was shown to improve pretreatment and current treatment of OP intoxication (Doctor and Saxena, 2005). However, in order to prevent abnormally fast pharmacokinetics and/or immunological response due to injection of a bacterial enzyme, PTE could be PEGylated (Jun et al., 2007) or encapsulated. The first attempts at using PTE encapsulated within sterically stabilized liposomes were promising, providing protection of rats from multiple LD_{50}s of OP pesticides (Petrikovics et al., 2004). An alternative route could be the blood detoxification by extracorporeal circulation through a cartridge containing PTE immobilized in hollow fibers (unpublished results). PTEs possibly could also be used for skin protection as active components of TSPs or covalently coupled to the cornified layer of epidermis (Parsa and Green, 2001).

B. diminuta PTE was also entrapped in additives for latex coating of biodefensive surfaces. Such PTE-based additives for paints and coatings were shown to retain catalytic parameters and stability of the enzyme (McDaniel et al., 2006). For decontamination of OPs in the environment and remediation, an alternative approach, phytodegradation by transgenic plants (e.g., tobacco) expressing a bacterial PTE, has been considered as a potentially low-cost, effective, and environmentally friendly method (Wang et al., 2008a,b). It should be mentioned that His-tagged PTE (Efremenko et al., 2009) was reported to degrade NAs, including VX, at a high rate. Since the enzyme was not genetically modified, it is suggested that the presence of the His tag plays a role in this amazing activity. Though neither 3D structure nor molecular dynamics (MD) studies are available for this enzyme, it can be hypothesized that the His tag may increase the enzyme flexibility, which in turn should increase the enzyme capability to accommodate numerous OP molecules and improve its catalytic activity.

Highly stable promiscuous lactonases/phosphotriesterases from hyperthermophilic archaea have been isolated in hot springs and volcano *solfatare*. The three-dimensional structures, evolution, stability, and catalytic properties of several PTEs have been determined (Elias et al., 2008; Hiblot et al., 2012, 2013a,b; Zhang et al., 2012; Gotthard et al., 2013; Porzio et al., 2013). These enzymes have been conveniently expressed in *E. coli* and

TABLE 72.1 Catalytic efficiency (k_{cat}/K_m, $M^{-1}min^{-1}$) of different natural and engineered OP hydrolases towards different Ops.

Source of enzyme	paraoxon	DFP	tabun (GA)	sarin (GD)	soman (GD)	cyclosarin (GF)	echothiophate	VX
Human PON1 Q192	6.8×10^5 [a]	4×10^4 [b]		9.1×10^5 [c]	2.8×10^6 [c]			+ [d]
Human PON1 R192	2.4×10^6 [a]			7×10^4 [c]	2.1×10^6 [c]			+ [d]
Human rPON1 in 293T					6.2×10^5-4.1×10^6 [e]			
Mammalian rPON1 G3C9	7.2×10^5 [f]							
Mammalian rPON1 V346A					8.7×10^4 [g]	3.6×10^5 [g]		
Chimeric rPON1 IIG1			2.6×10^6 [h]	9.5×10^6 [h]	6.4×10^7 [h]	8.4×10^7 [h]		1.43×10^2(*) [h]
Human BChE G117H	5.7×10^3 [i]	5.2×10^3.[i]		1.6×10^2 [j]	–		1×10^4 [i]	1.5×10^3 [j]
Blowfly CaE G117D	2×10^5 [k]							
Human CaE1 (V146H/L363E) *B. fasciatus*AChE HQT	64 [i]	7.6×10^2 [i]				5.3×10^3 [l]	24 [h]	
*Loligo vulg*aris DFPase		7.8×10^7 [m]		2.4 $\times10^6$ [m]	2.4×10^6 [m]			0 [m]
P. diminuta PTE	2×10^9 [n]	5.8×10^8 [o]		4.8 $\times10^6$ [p]	6×10^5 [p]	5×10^3 [q]		4×10^4 [r]
*P. diminuta*PTE (H257Y/L303T) *Alteromonas*sp. JD6.5OPAA		44.6×10^7 [s]		12×10^7 [t]	3×10^6 [t] 14.6 [r]	4.8 $\times10^6$ [t]		
*Alteromonas*sp. JD6.5cloned				5.8×10^6 [u]	1 $\times$ 10^7 [u]	6.2×10^7 [u]		
Alteromonasundina			21.8 [v]	30.4 [v]	1.6×10^2 [v]	1.3×10^2 [v]		
Sulfolobussolfataricus (W263F) PTE NG108-15 hybrid cells	7.2×10^5 [w]				2.5×10^3 [x]			

[a] *(Smolen et al., 1991)*.
[b] *(Masson et al., 1998)*.
[c] *(Davies et al., 1996)*.
[d] *C.A. Broomfield, unpublished result.*
[e] *(Yeung et al., 2008), with the four soman stereoisomers.*
[f] *(Harel et al., 2004)*.
[g] *(Amitai et al., 2006)*.
[h] *(Worek et al., 2014) (*, under second order conditions)*.
[i] *(Poyot et al., 2006)*.
[j] *(Lockridge et al., 1997)*.
[k] *(Newcomb et al., 1997)*.
[l] *(Hemmert et al., 2011)*.
[m] *(Hartleib and Rüterjans, 2001)*.
[n] *(Kuo et al., 1997)*.
[o] *(Lai et al., 1995)*.
[p] *(Dumas et al., 1990)*.
[q] *(Hoskin et al., 1995)*.
[r] *(Rastogi et al., 1997)*.
[s] *(Cheng et al., 1999)*.
[t] *(Tsai et al., 2012)*.
[u] *(Cheng et al., 1996)*.
[v] *(DeFrank et al., 1993)*.
[w] *(Hiblot et al., 2013a,b)*.
[x] *(Ray et al., 1988)*.

mutated (site-directed mutagenesis and directed evolution) to improve their catalytic properties against OPs (Merone et al., 2010; Hiblot et al., 2012, 2013a,b; Jacquet et al., 2017; Restaino et al., 2017) (Table 72.1). Owing to their high stability that allows long-term storage at room temperature, fieldable uses for decontamination are possible. Other extremophile PTEs have been isolated from halophilic bacteria (*Alteromonas*), such as the OPAA (organophosphorus acid anhydrolase) and from radio-resistant bacteria, *Deinococcus radiodurans* and *A. radiobacter*. The 3D structure and catalytic mechanism of these enzymes have also been determined and used for structure-based random mutagenesis rational design to improve their catalytic efficiency against OPs (Hawwa et al., 2009; Jackson et al., 2009; Ely et al., 2010). Recent mutagenesis of OPAA generated new mutants against G agents, one of these mutants displays the highest activity against soman (Bae et al., 2018). New fluorimetric screening methods allow identification of highly active PTEs in microorganisms from various biotopes (Santillan et al., 2016).

72.6.1.2 Human paraoxonase

The human paraoxonase-1 (PON-1) is a 45 kDa calcium-dependent plasma enzyme bound to high-density lipoprotein (HDL) particles, in association with other apolipoprotein partners. PON-1 shows a genetic polymorphism; the most prominent allele determines the Q192R allozyme, which can have a substantial impact on PON-1 activity against OPs and arylesters (Smolen et al., 1991; Dardiotis et al., 2019) (Table 72.1). The enzyme was shown to be involved in the protection against atherosclerosis (Watson et al., 1995; Shih et al., 1998) and thus became a player in vascular physiology. Albeit its primary function is a lipophilic lactonase (Khersonsky and Tawfik, 2005; Ben-David et al., 2013), PON-1 displays two promiscuous activities, PTE and arylesterase (Blaha-Nelson et al., 2017).

Abundant biochemical, biological, and toxicological information has been collected in the past two decades, leading to partial characterization of the enzyme function (Costa and Furlong, 2002; Mackness et al., 2008), but recurrent attempts at solving the 3D structure of human PON-1 failed. Through chemical modification and site-directed mutagenesis studies, some amino acid residues have been identified as essential for activity (Josse et al., 1999). Finally, molecular modeling (Fokine et al., 2003; Yeung et al., 2004) and crystal structure of a hybrid rPON-1 (a synthetic construct issued from shuffling of rabbit, mouse, rat, and human PON-1 genes expressed in *E. coli*) (Harel et al., 2004) showed that human PON-1 is a six-bladed β-propeller protein with a structure very similar to that of *Loligo vulgaris* DFPase (Katsemi et al., 2005). Differences in substrate preference between PON-1 and DFPase reflect the molecular evolution of these related enzymes (Zhang et al., 2018).

A catalytic mechanism for DFPase, a calcium-dependent PTE, was described (Blum et al., 2006; Bigley and Raushel, 2013). This mechanism involves a calcium-coordinated aspartate residue as the nucleophile that attacks the OP phosphorus atom. Alternatively, a more realistic mechanism involves the activation of a water molecule into a hydroxide ion that attacks the phosphorus center (Elias et al., 2013). This mechanism is consistent with the common mechanism that has been proposed for PON-1 (Ben-David et al., 2013), PTEs (Aubert et al., 2004), and PLLs (Elias et al., 2008). Lastly, a unified mechanism for eukaryotic PTEs was recently proposed (Grunkemeyer et al., 2018). This mechanism considers that the water molecule is activated by a calcium cation. Hydroxyl ion either directly attacks the substrate (carbonyl or phosphoryl group, depending on the type of substrate), or activates another nearby water molecule. Surrounding residues play an important role in modulating activity, but mutagenesis results suggest that they are not involved directly (Grunkemeyer et al., 2018). This mechanism is close to the above-mentioned mechanism for bacterial PTEs containing two divalent metal cations (Bigley and Raushel, 2019) (Fig. 72.3C).

As a naturally occurring enzyme present in plasma, human PON-1 has been considered as the most promising catalytic bioscavenger candidate for pretreatment and therapy of poisoning by OP (La Du, 1996; Rochu et al., 2007a; Worek et al., 2014). Thus, the enzyme has been the focus of intensive research to improve its efficacy and functionalization. To provide a valuable medical countermeasure against intoxication by NAs, the catalytic efficiency of PON-1 has to be enhanced by only one or two orders of magnitude. Chimeric PON-1 mutants obtained by directed evolution and exhibiting enhanced OP-hydrolase activity (Amitai et al., 2006; Ashani et al., 2011; Worek et al., 2014) show this goal to be reasonably achieved soon. However, instability of PON-1 mutants could impinge on their biotechnological development. Indeed, as a HDL-bound protein, PON-1 requires association to apolipoprotein partners to retain its stable active conformation (James and Deakin, 2004; Gaidukov and Tawfik, 2005; Rochu et al., 2010) and long residence time in the bloodstream (Valiyaveettil et al., 2012). Highly purified human plasma PON-1 was found to be associated to human Phosphate Binding Protein (HPBP) (Rochu et al., 2007b). HPBP is an apolipoprotein that binds inorganic phosphate in blood. HPBP was serendipitously discovered (Morales et al., 2006). This protein belongs to the family of DING proteins (Berna et al., 2009). Its three-dimensional structure and complete amino acid sequence were solved (Morales et al., 2006; Diemer

et al., 2008). The conditions found to separate HPBP and PON1 in vitro indicated that HPBP is strongly associated with PON-1 (Renault et al., 2006). Moreover, stabilization of the active form(s) of human PON-1 by HPBP suggests that HPBP could be a functional chaperone for PON1 (Rochu et al., 2007b,c; Clery-Barraud et al., 2009). Biotechnological difficulties encountered with plasma-derived human PON-1 have been overcome by expressing the enzyme in a stably *Drosophila* S2 cell line. The recombinant human PON-1 was fused to the human immunoglobulin Fc domain to improve stability and purification (Yun et al., 2017a,b).

A gene issued from the HPBP amino acid sequence was synthesized (Diemer et al., 2008), and the protein was expressed in *E. coli*. However, attempts at cocrystallization of the PON-1−HPBP complex have failed so far. Yet this crucial phase is the first step of the staircase leading to the design and development of stable human PON-1 mutants with enhanced catalytic efficiency against toxic OP stereoisomers (S_p) of G NAs.

Site-directed mutagenesis of human PON-1 based on molecular modeling has led to double mutants capable of hydrolyzing G and V NAs (Kirby et al., 2013). However, the catalytic efficiency of these mutants is modest and enantioselectivity is not changed compared to the wild-type enzyme. A directed evolution strategy has been more successful in producing chimeric PON-1 capable of degrading toxic isomers of coumarinyl NA analogs as well as real G-NAs. In particular, enantioselectivity of evolved PON-1 was completely reversed. For instance, the activity against S_p-cyclosarin was enhanced 10^5-fold (Ashani et al., 2011; Gupta et al., 2011; Goldsmith et al., 2012). The most active evolved PON-1 mutant, IIG1 (Table 72.1), administered at a dose of 1 mg/kg was shown to prevent $2 \times LD_{50}$ cyclosarin systemic toxicity in the guinea pig (Worek et al., 2014). This mutant was also shown to detoxify most G-NAs at a high rate under first-order conditions, but was ineffective against VX even under second-order conditions (Goldsmith and Ashani, 2018).

Gene therapy could also be considered to challenge OPs by using a mutated PON-1 gene coding for an enzyme with high OPH activity against NAs. Several approaches with different gene-delivery vectors in mice showed increased PON-1 level serum that reduced or even prevented the entry of OP into the brain, and reduced atherosclerosis signs (Cowan et al., 2001; Bradshaw et al., 2005; Fu et al., 2005; Guns et al., 2008; Duysen et al., 2011; Hodgins et al., 2013). Local delivery of PON-1 gene using Sendai virus vector inhibited neonatal hyperplasia after arterial balloon-injury in rabbits fed a high-fat diet (Miyoshi et al., 2007). However, wild-type human PON-1 does not have sufficient catalytic efficiency against NAs to provide in vivo protection against $2 \times LD_{50}$ of G agents. Thus, enhanced expression of mutated PON-1 by gene therapy could be beneficial for the different functions of the enzyme. Meanwhile, the complex and defectively identified PON-1 activity makes it apparent that the strategy for repetitive administration of high concentrations in humans must be undertaken cautiously.

72.6.2 Other enzymes

Other enzymes are involved in biodegradation of OPs; including hydrolases, for example, prolidases, senescence marker protein (SMP), and platelet-activating factor (PAF-AH); others are oxidases, such as cytochromes P450, GSTs, laccases, and peroxidases.

72.6.2.1 Other mammalian phosphotriesterases

Prolidases (EC 3.4.13.9, PROL) were first isolated from halophilic bacteria (*Alteromonas haloplanktis* and *Alteromonas* sp. JD6.5). This bacterial metallo-enzyme (binuclear Mn^{2+} center) has a "pita bread" structure (Vyas et al., 2010). Prolidase from *Alteromonas* sp. JD6.5 is an OPAA that displays high activity against soman ($k_{cat} = 3100\ s^{-1}$), but it is inactive against VX (Cheng et al., 1999). Thermostable prolidases from hyperthermophilic archaeas *Pyrococcus furiosus* (Theriot et al., 2010) and *Pyrococcus horikoshii* (Theriot et al., 2011) hydrolyze P−F and P−O bonds in NAs. Evolved mutants of these enzymes capable of degrading OPs over wide temperature ranges, as engineered hyperthermophilic PLLs do, should have a future for biodecontamination under mild conditions. PROL was also isolated from human liver, kidney, erythrocytes, and skin and expressed in *E. coli* (diTargiani et al., 2010; Costante et al., 2012; Chandrasekaran et al., 2013). This enzyme displays a catalytic activity against sarin and soman (Wang et al., 1998; diTargiani et al., 2010) and exhibits sequence homology with the *A. haloplanktis* prolidase (Wang et al., 2006). First engineered human PROL showed that mutants A265R and P365R have a slightly improved activity against DFP (Yun et al., 2017a,b). Also, gene-delivered human liver PROL using adenovirus produced a high level of active enzyme, but protected mice only against $1 \times LD_{50}$ of DFP (Aleti et al., 2013).

SMP-30 was isolated first from rat liver (Kondo et al., 2004). It is a six-bladed-β-propeller metallo-lactonase structurally close to PON-1 and DFPase with a promiscuous PTE activity (Chakraborti and Bahnson, 2010). The human and mouse enzymes display a modest PTE against G-agents (diTargiani et al., 2010; Belinskaya et al., 2012). Engineering of this enzyme is still at the early stages.

PAF-AH is a group of 45 kDa lipoprotein (HDL and LDL)-associated phospholipases A2. In human, they are present both in plasma and brain. These enzymes that

react with numerous OPs are structurally related to NTE (Karasawa et al., 2003; Quistad et al., 2003, 2005; Epstein et al., 2009). Site-directed mutagenesis of human plasma PAF-AH for making an OPH with the purpose of NA detoxification has been undertaken (Kirby et al., 2012). However, research on these enzymes as possible catalytic bioscavengers is still in its infancy.

72.6.2.2 Oxidases

GSTs (EC 2.5.1.18) are 20–30 kDa enzymes that catalyze glutathione conjugation (nucleophilic attack of the thiol group) to electrophilic substrates. They are involved in cellular detoxification processes of endogenous compounds and of numerous xenobiotics, and their role in insect resistance to insecticides is well established. OP detoxification by GSTs results from a regioselective dealkylation of the alkyl or aryl side chain (Maturano et al., 1997). These enzymes exhibit wide genetic polymorphism. Some GST allelozymes from the flies *Drosophila melanogaster* and *Musca domestica*, highly active against OP insecticides, have been cloned and expressed in *E. coli* (Fournier et al., 1992). There is evidence that GSTs contribute to OP detoxification in humans (Fujioka and Casida, 2007).

Laccases (EC 1.10.3.2) are fungal phenol oxidoreductases that have been used for detoxification of numerous xenobiotics, including dyes and pesticides (Richardt and Blum, 2008). The laccases from *Pleurotus ostreatus* and *Chaetomium thermophilium* were found to rapidly degrade VX and VR in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as a mediator (Amitai et al., 1998). Laccases from *Trametes versicolor* and *Coriolopsis polyzona* with ABTS display similar properties against V agents (Trovaslet-Leroy et al., 2010). The heme-containing chloroperoxidase (EC 1.11.1.X) from *Caldaromyces fumago*, with peroxide as cosubstrate, is another efficient VX-degrading enzyme (Amitai et al., 2003). A bacterial laccase from *Pseudomonas* sp. S2 produced in a bioreactor was found to oxidize OP pesticides in a short time (Chauhan and Jha, 2018). This enzyme could at least be used for decontamination.

These enzymes are promising for the destruction of chemical weapons stockpiles, soil remediation, and the decontamination of materials, protective equipment, and water polluted by pesticides and NAs (Russell et al., 2003). In particular, phosphorothiolates such as VX are almost resistant to PTEs. Thus, oxidative cleavage of the P–S bond could be achieved by oxidases like laccases. These enzymes could be used in association with other OP-degrading enzymes for skin decontamination or in topical skin protection formulations. Though no work has been performed on the combined action of oxidases and hydrolases, oxidation of P-bonded alkyl/aryl chains by oxidases is expected to alter the enantioselectivity of PTE for parent OPs. Therefore, biopharmaceutical formulations of combined oxidases and PTEs may improve the efficiency of PTE-based catalytic bioscavengers.

72.6.3 Engineered cholinesterases and carboxylesterases

As seen in Fig. 72.1, OPs may be regarded as pseudo-substrates of cholinesterases and carboxylesterases. When ChEs and CaEs react with carboxyl-ester substrates, the acyl-enzyme intermediate is a transient, the acyl group being rapidly displaced by a water molecule. On the contrary, in the case of phosphyl-esters, the stereochemistry of the phosphyl-enzyme intermediate restricts the accessibility of water to the phosphorus atom. Thus, hydrolysis of the phosphylated intermediate is very slow, and the enzyme remains inhibited (Järv, 1989). It was postulated that introduction of a second nucleophile pole in the active center could activate a water molecule. This water molecule could subsequently attack the phosphorus atom on the back face, leading to breakage of the P-serine bond.

The determination of the three-dimensional structure of AChE from *Torpedo californica* (Sussman et al., 1991) opened the way to rational redesign of ChEs. Then, the possibility to convert a ChE into an OPH was demonstrated a few years later by Millard et al. (1995). Human BChE was chosen as the model enzyme because its active center is larger (500 $Å^3$) and less stereospecific than that of AChE (300 $Å^3$) (Saxena et al., 1997). Molecular modeling based on the structure of the *Torpedo* AChE model was used for making the first mutants of human BChE. The second nucleophile pole was created in the oxyanion hole of the active center; a glycine residue was replaced by a histidine. The first mutant, G117H, was capable of hydrolyzing paraoxon, sarin, echothiophate, and VX (Millard et al., 1995; Lockridge et al., 1997) (Fig. 72.4). However, this mutant was irreversibly inhibited by soman because the "aging" process of the conjugate was faster than the dephosphonylation reaction (Fig. 72.1, reaction 4). Mutation of E197 into D, Q, or G considerably reduced the rate of aging. As expected, the double mutant G117H/E197Q was capable of hydrolyzing soman (Millard et al., 1998). However, the catalytic activity of this mutant was too slow to be of pharmacological interest.

Construction of transgenic mice *knockout* for AChE and carrying the G117H mutant of human BChE were found to be less sensitive to OP than wild-type animals (Wang et al., 2004). Though transgenic mice expressed G117H mutant in all organs, unlike resistance of the bow fly, their resistance to OP cannot be explained by OP

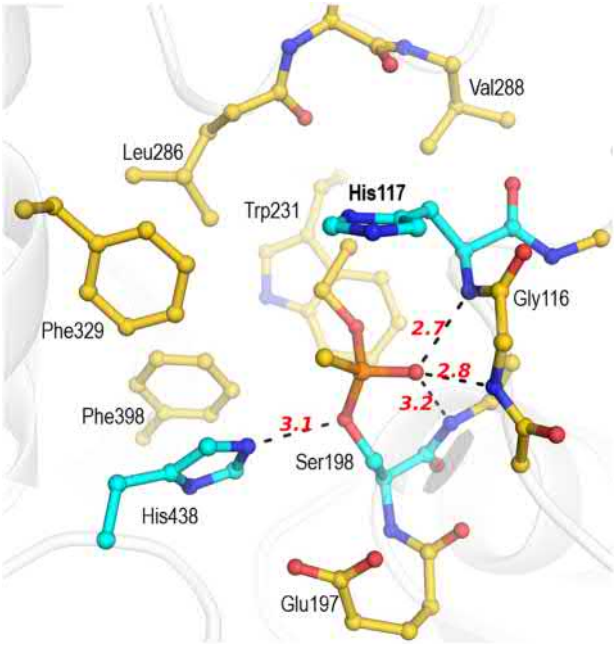

FIGURE 72.4 X-ray structure of the active center of G117H mutant of human butyrylcholinesterase conjugated with VX at 2.1 Å resolution (Nachon et al., 2011).
The enzyme was phosphorylated by soaking the crystal into 1 mM VX for 2 min. X-ray data were collected at European Synchrotron Research Facility (ESRF, Grenoble, France). Two catalytic triad residues, active serine (S198) and histidine (H438), and the mutated residue (H117) in the oxyanion hole are shown as sticks as well as other active center important residues.

hydrolysis that was too slow, but rather by hydrolysis of excess acetylcholine in cholinergic synapses.

More than 60 double or triple mutants based on G117H were made (Schopfer et al., 2004; Lushchekina et al., 2018), certain mutants were eventually designed using the X-ray structure of human BChE (Nicolet et al., 2003). At the same time, mutants of human AChE and *Bungarus fasciatus* AChE were made, using the same strategy (Poyot et al., 2006). Unfortunately, none of these muteins was more active than the G117H mutants. Actually, there is evidence that mutations at position G117 cause dislocation and loss of functionality of the oxyanion hole (Masson et al., 2007). For a historical review of this quest, see Masson et al. (2008).

The crystal structure of the G117H mutant conjugated to echothiophate and VX was solved (Nachon et al., 2011) (Fig. 72.4). Despite numerous works, the mechanism of dephosphylation of this mutant is still debated (Amitay and Shurki, 2011; Lushchekina et al., 2011; Nachon et al., 2011; Yao et al., 2012; Masson and Lushchekina, 2016; Lushchekina and Masson, 2018). However, computer-assisted design of new OPH mutants of BChE is conceivable (Lushchekina et al., 2010, 2018; Grigorenko et al., 2019). This new approach called "intelligent" directed mutagenesis design is based on simulation of the reaction mechanism. Simulation of the reaction pathway approach was already successfully applied to the design of other BChE mutants. Using the three-dimensional structure of human BChE, modeling of intermediate and transition state structures with QM and QM/MM calculations along deacylation reaction coordinates allowed highly active mutants against (−)-cocaine to be made (Zheng and Zhan, 2008; Liu and Zhan, 2012; Zhan et al., 2014). A computational strategy for developing OPH BChE mutants, more active than G117H, aims at optimizing the dephosphylation reaction pathway in the following ways: (1) lowering the energy barrier, that is, the energy level of transition states of the reactions to accelerate kinetics of the process; (2) stabilizing the intermediate and products to favor thermodynamics of the process; and (3) creating an intermediate configuration more favorable for a dephosphylation reaction rather than dealkylation (aging).

Structurally, this is based on the principles, initially used for creation of G117H mutant (Järv, 1989). (1) Accessibility of the phosphylated serine by water molecules. The environment of the phosphylated adduct is highly hydrophobic, it is surrounded by F398, F329, W231, L286 (Fig. 72.4). Natural BChEs, capable of spontaneous self-reactivation [bovine BChE (Dafferner et al., 2017) and porcine BChE (Brazzolotto et al., 2015; Nachon et al., 2019)] have substitutions in the acyl-binding loop compared to the wild-type human enzyme. In addition, in bovine BChE, a bulky aromatic residue, F398, is replaced by I, a smaller aliphatic residue. MD simulations showed that mutations in the acyl-binding loop increase its mobility. This improves the accessibility of phosphylated serine by water molecules (Brazzolotto et al., 2015; Dafferner et al., 2017). Creating-space replacements, like F398I and F329E, alone do not lead to self-reactivation; the self-reactivating effect results from its coupling to mutations in the acyl-binding loop (Lushchekina et al., 2018). (2) Activation of the water molecule by general base (nucleophile pole) can be performed either by residues present in the active site of native BChE (H438, E197), or by introduction of new residues instead of "inert" ones. Additional mutations can be introduced to facilitate proton transfer in the course of water activation, and thus, stabilize the resultant intermediate. The water-activating potency of residue strongly depends on its protonation state in the active site (Lushchekina and Masson, 2018). (3) Orientation of the water molecule can be changed by introduction of general bases in corresponding positions. For the pentavalent phosphorus conjugates there are four possibilities. Choice of direction determines both the route of nucleophilic attack and the configuration of the penta-coordinated intermediate (PI), and consequently, the results of

competition between reactivation and aging reactions. Therefore, understanding mechanisms of interactions between ChEs and OPs is the foundation for the design of novel mutants. The development of structural and computational methods led to ample amount of works, describing all processes, as shown in Fig. 72.1. The different mechanisms are summarized in Lushchekina and Masson (2018),

72.6.3.1 Inhibition of cholinesterases by organophosphates

The formation of a reversible complex between enzyme and inhibitor (Fig. 72.1, step 1) can be accompanied by conformational changes (Bennion et al., 2015); this is well documented in the case of bulky noncovalent inhibitors. In the case of OPs, orientation of the OP molecule in the active site determines the mechanism of covalent reaction (Fig. 72.1, step 2). There are three possible mechanisms of phosphyl transfer between two nucleophiles: concerted (A_ND_N), dissociative ($D_N + A_N$), and addition-elimination or associative ($A_N + D_N$) (Allen and Dunaway-Mariano, 2004). However, for inhibition of ChEs by OPs, the addition-elimination mechanism with formation of stable penta-coordinated intermediate is considered the most probable (Kovach, 1988; Wang et al., 2008a,b; Field and Wymore, 2014; Sirin and Zhang, 2014). Upon formation of PI, the proton of the catalytic serine is transferred to the catalytic histidine. Within the addition-elimination mechanism, there are two possible pathways, depending on orientation of the OP molecule during the nucleophilic attack (Fig. 72.5). (1) leaving group in apical position to the catalytic serine (Fig. 72.5, route A and Fig. 72.6A). After backside nucleophilic attack, and formation of PI, the leaving group departs in anionic form. The catalytic histidine remains protonated in the phosphylated enzyme. This mechanism leads to inversion of configuration of the chiral phosphorus atom for OPs with different substituents. (2) Leaving group in side orientation (Fig. 72.5, route B and Fig. 72.6B), with respect to catalytic residues. The leaving group departs in protonated form with proton abstracted from the catalytic histidine during dissociation of PI. In this case, the stereoconfiguration of the phosphorus atom is retained.

In general, nucleophilic substitution for pentavalent phosphorus proceeds with inversion of configuration via the backside mechanism (Kolodiazhnyi and Kolodiazhna, 2017). However, echothiophate, one of the most popular OP models with a nonchiral phosphorus atom, has the same thiocholine-leaving group as thioster substrates (acetyl/butyryl-thiocholine) (Fig. 72.6C). In enzyme−substrate complexes, the thiocholine moiety is bound to the cation-binding pocket formed by W82 and E197. For echothiophate, such a position for the leaving group leads to a sideway attack and inhibition reaction according to mechanism Fig. 72.4B.

The mechanism with a leaving group in the apical position from serine was elaborated by Kovach and her coworkers (Kovach, 1988, 2004; Qian and Kovach, 1993; Kovach et al., 1997), similar schemes are presented in Beck and Hadad (2010), Field and Wymore (2014), and Bennion et al. (2015). Docking of VX into AChE showed that the leaving group is opposite to the catalytic serine, while it is in side orientation in BChE (Bennion et al.,

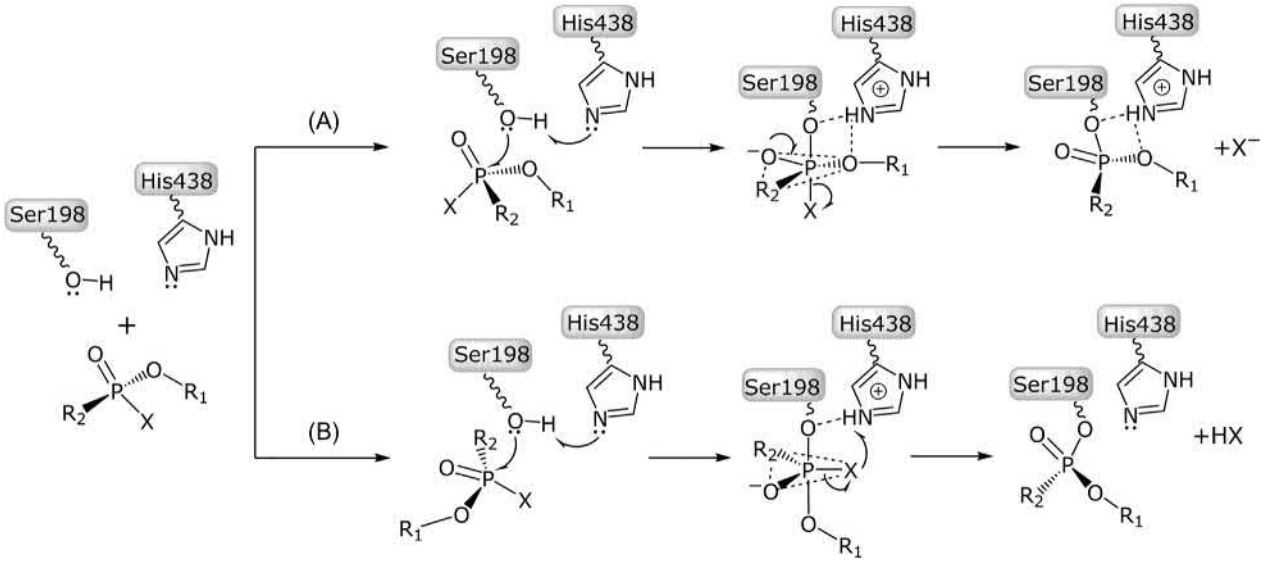

FIGURE 72.5 Mechanism of ChE inhibition by OPs. (A) Binding of inhibitor with its leaving group opposite to the catalytic serine leads to protonated histidine in phosphylated adduct. (B) Binding of inhibitor with the leaving group aside from catalytic serine and histidine suggests that histidine may be deprotonated after departure of protonated leaving group.

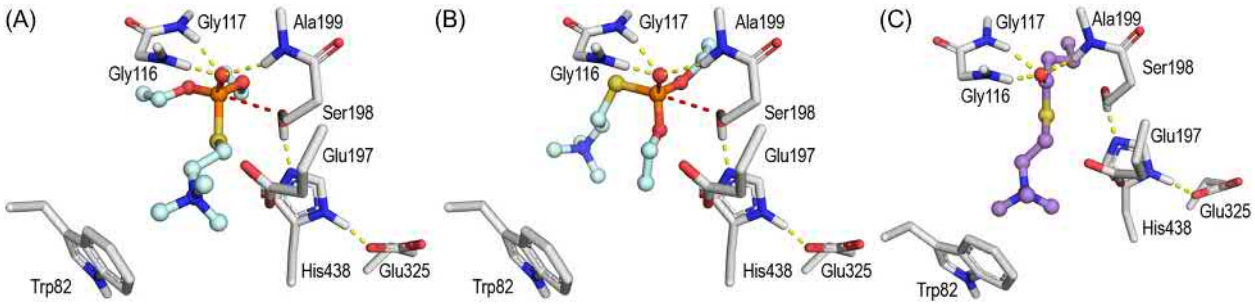

FIGURE 72.6 Possible positions of echothiopate in BChE active site in enzyme inhibitor complex for: (A) backside nucleophilic attack, and (B) side attack. (C) Enzyme−substrate complex BChE/butyrylthiocholine.

2013). This suggests a mechanism backside nucleophilic attack for AChE and a side attack for BChE. Detailed reaction schemes of AChE phosphorylation (Liu et al., 2009; Quinn et al., 2017) specifically show the leaving group in protonated form and histidine deprotonated, implying the side position of the leaving group.

Recent computational studies (Zlobin et al., 2018a,b; Grigorenko et al., 2019) directly compared these two mechanisms, taking into account the different OP conformations. These studies indicate that the leaving group is in an apical position outside the cation-binding site, an orientation different from substrate in the active center, and more favorable for inhibition reaction. This is consistent with inversion of the configuration of the phosphorus chiral center for reaction of BChE as observed with optically pure VX enantiomers (Wandhammer et al., 2011) and with early observations about reactions of OPs with AChE (Ashani et al., 1973). This establishes that in inhibited enzyme catalytic histidine is protonated, and hence cannot activate water for a dephosphylation/delakylation reaction, but rather orients this water molecule, unless a proton transfer events take place.

72.6.3.2 Proton transfer wires

The protonated state of the catalytic histidine in phosphorylated conjugate suggests that the noncatalytic glutamic acid is the nucleophilic pole, activating a water molecule for spontaneous reactivation or aging reactions. This statement is supported by the fact that mutation of E197 into D, Q, or G considerably reduces the rate of aging (Masson et al., 2008). However, another point of view that E197 (E199 in *Tc*AChE, E202 in hAChE) is protonated has been persistent in the literature since the X-ray structure of *T. californica* AChE was solved (Soreq et al., 1992). E197 is coupled with E441 through bridging of a water molecule. Lack of proton between E197 and E441 may lead to artifacts in modeling due to electrostatic repulsion between two negatively charged amino acids and unsaturated hydrogen bonding. In MD simulations of di-isopropyl-phosphorylated BChE, E441 was considered protonated (Masson et al., 1997), some further MD simulations of BChE and its mutants followed this approach (Sun et al., 2001; Suarez and Field, 2005). EVB modeling of the catalytic reaction in G117H BChE mutant (Amitay and Shurki, 2009) suggested that protonation of E197 reduces the reaction energy barrier. It was observed that AChE in MD simulations is more stable when one or two of these residues are protonated (Wiesner et al., 2010). Detailed QM/MM study of the protonation state of E202 during oxime-mediated reactivation of VX-inhibited ACHE was published recently (Driant et al., 2017); referring to this work further studies mention the unusually high pK_a of E197 in human BChE (Rosenberry et al., 2017) and E202 in human AChE (de Koning et al., 2017; Rosenberry et al., 2017). Later, the possibility of protonated E197 was rediscovered in an MD study of the tacrine−BChE complex (Wan et al., 2018).

QM/MM calculations of proton transfer processes in diethylphosphorylated BChE showed that protonated catalytic histidine may transfer its proton to E197 through a chain of water molecules. Proton may be transferred from E197 to E441 and back, through bridging water molecule, depending on the charge distribution in the active site (Lushchekina and Masson, 2018) (Fig. 72.7). Hence, both H438 and E197 may serve as a general base activating and orienting water molecules for reactivation and aging reactions.

72.6.3.3 Mechanism of aging

Possible mechanisms of aging (Fig. 72.1, step 4) of soman-aged ChEs were actively debated before resolution of the X-ray structure of AChE (Kovach, 1988), shortly after (Kovach et al., 1993), and in the following years, with new crystallographic and kinetic data for various mutants (Shafferman et al., 1996, 1997; Kovach et al., 1997; Viragh et al., 1997; Saxena et al., 1998a,b). Later, when the X-ray structures of aged and nonaged forms of soman-inhibited *Tc*AChE were obtained, the aging mechanism was clarified (Sanson et al., 2009), and QM/MM modeling of this reaction was performed (Sirin et al.,

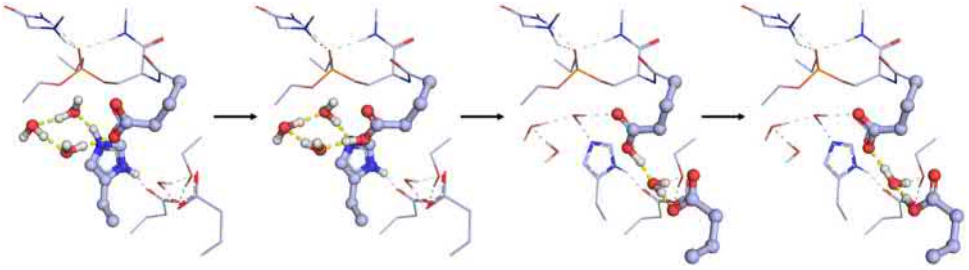

FIGURE 72.7 Concerted proton transfer from protonated H438 to E197 through surrounding water molecules and to E441 through bridging water molecule.

2012). Markedly, it was found that this reaction is favorable when E199 is protonated. Generally, aging of soman-inhibited AChE is thought to occur through formation of planar pinacolyl carbenium ion, which undergoes rearrangement into a mixture of 2,3-dimethyl-2-butanol, 2,3-dimethyl-1-butene, and 2,3-dimethyl-2-butene.

Something rather different was observed in the X-ray structure of echothiophate-inhibited human BChE (Nachon et al., 2005a,b). The resolved water molecule is able to form hydrogen bonds with H438 and E197. Assuming H438 protonated, the water molecule is activated by E197, and vice versa. After formation of penta-coordinated intermediate aging is induced via breaking either a C−O bond or a P−O bond (Fig. 72.8). Mass-spectrometry analysis of BChE aged after phosphorylation by echothiophate in the presence of H_2O^{18}, suggested that aging proceeds through C−O bond cleavage for different OPs (Li et al., 2007).

72.6.3.4 Spontaneous reactivation

There are numbers of crystallographic and theoretical studies on oxime-assisted reactivation (Fig. 72.1, step 3), while studies of spontaneous reactivation of phosphylated ChEs are few. For most OP adducts, self-reactivation is much slower than aging, or it cannot be recorded experimentally. However, molecular modeling can be used to force a system through an energetically unfavorable pathway to examine the possible reaction mechanism. Such an approach allows comparison of energy barriers for wild-type enzymes and suggests mutations for designing novel ChE-based catalytic bioscavengers.

Suggested aging mechanisms through P−O bond cleavage can also be applied for spontaneous reactivation mechanisms with PI dissociation through proton transfer from H438 to S198 oxygen atom (Fig. 72.8).

The X-ray structure of the G117H BChE mutant was published in 2011 (Nachon et al., 2011). Four possible mechanisms for the role of H117 in self-reactivation were discussed according to crystallographic data. Activation of a water molecule by H117 was a dominating idea, but the X-ray structure revealed that there is not enough room for water molecules to approach the phosphorus atom from the side of H117. It was concluded that the role of H117 in catalytic reactivation is mostly to cause geometric disturbance in the oxyanion hole and phosphoryl adduct. In computational studies of the spontaneous reactivation mechanism for diethylphosphorylated G117H BChE mutant, discussed below, energy profiles for the same reaction in wild-type were computed, with either protonated H438 (Kulakova et al., 2015; Masson and Lushchekina, 2016; Nemukhin et al., 2016) (Fig. 72.8A) or not protonated (Yao et al., 2012) (Fig. 72.8B).

One of the emerging questions is the fate of the proton from the water molecule attached to the phosphorus atoms. Usually, in simulations of the reactivation reaction, it is left in hydroxyl group form. However, the product of diethylphosphorylated ChE reactivation is diethyl phosphoric acid with $pK_a = 1.39$ (Kumler and Eiler, 1943); and the product of reactivation of VX-inhibited ChE is ethyl methylphosphonate, $pK_a = 2.16$ (Richardson et al., 2006). Therefore, this proton has to be transferred either to catalytic histidine, or to a water molecule, forming a hydronium ion. For comparison, the mechanism of OP hydrolysis by bacterial PTE, discussed above, includes proton transfer to one of the vicinal residues upon hydrolysis product formation (Bigley and Raushel, 2019).

72.6.3.5 New routes of reactivation

Activation of the water molecule by H438 or E197 present in native BChE is only one of the possible directions of nucleophilic attack of the water molecule on the phosphorus atom. Other directions have been discussed in a recent work (Lushchekina et al., 2018). Residues L286, F329, F398, and W231 surrounding phosphylated serine (Fig. 72.4) are perspective places for replacement with a nucleophilic pole, for example, histidine. The histidine pK_a value is higher than the pK_a of aspartic and glutamic acids. Therefore, histidine can be protonated more easily, while those carboxy-acids remain options. This creates new directions for attack of a water molecule from three other sides (Fig. 72.9).

Direction A, adjacent to the serine residue, corresponds to the above-discussed mechanisms of aging and reactivation in wild-type and G117H BChE. Direction B from the apical position (opposite to the serine residue) can be realized with certain geometrical strains through

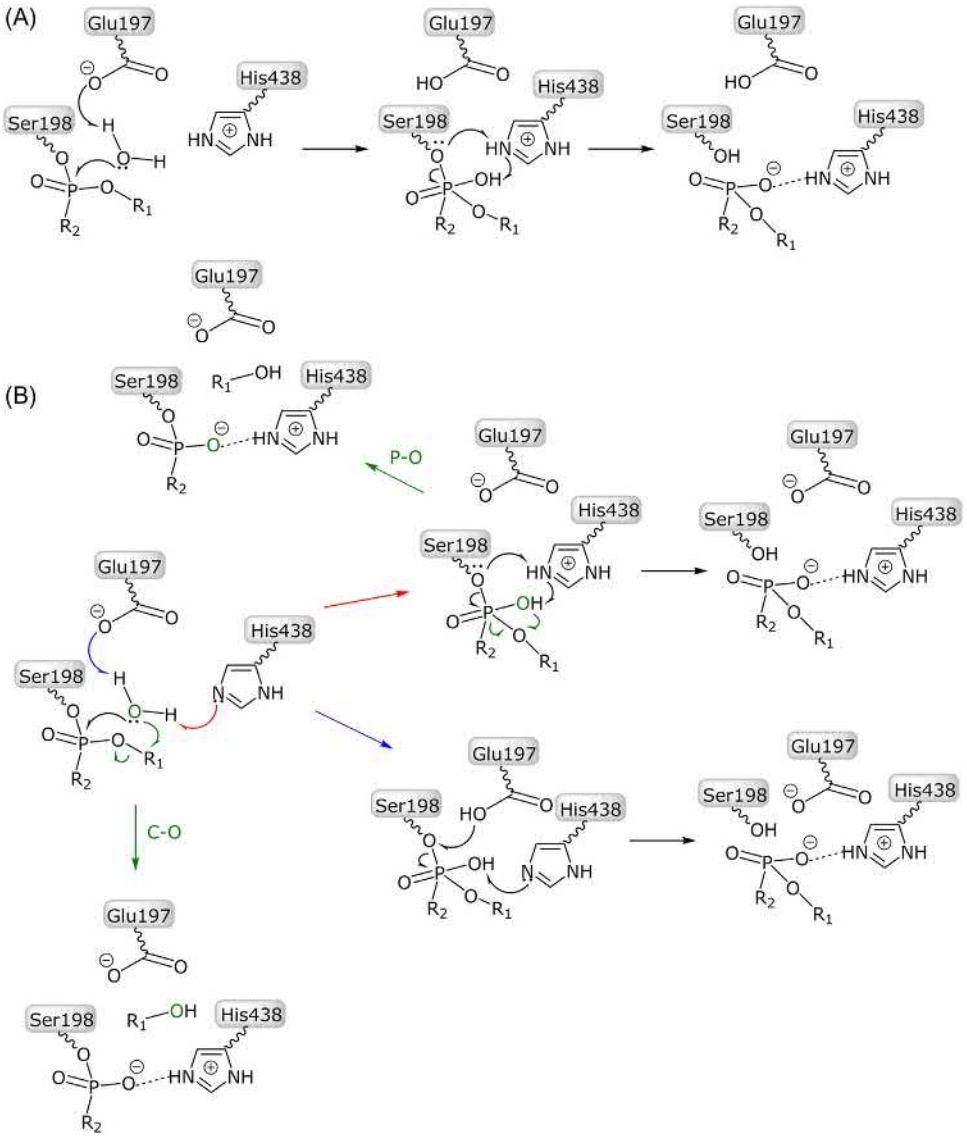

FIGURE 72.8 Possible mechanisms of spontaneous reactivation and aging: (A) with protonated catalytic histidine; (B) with nonprotonated histidine, red arrow—catalytic histidine activates water molecule, blue arrow—not catalytic glutamic acid activates the water molecule and transfers proton to release catalytic serine. Green arrows show aging mechanisms through P−O and C−O bond cleavage. Green oxygen atom shows position of O^{18} for the two pathways, studied by NMR in Li et al. (2007).

introduction of a histidine into position 286 of the acyl-binding loop. In the resulting PI, protonated H286 would form a hydrogen bond with an oxy-ethyl group from the diethylphosphate, suggesting strong competition between reactivation and aging processes. Direction C can be realized by introducing a histidine in position 398 and/or 329. Direction D can be achieved by replacement of W231, but might be ineffective alone due to poor accessibility for the water molecule.

Mutation L286H resulted in reactivation at a low rate ($k_3 \sim 0.0032\ \text{min}^{-1}$) (Schopfer et al., 2004; Lushchekina et al., 2018). QM/MM calculations show that the energy barrier for this mutant is slightly lower than that for G117H, but the PI is unstable because W231 hinders the shift of the diethylphosphate oxy-ethyl group in the trigonal bipyramid plane (Fig. 72.9). Replacement of the neighboring rigid P285 with a more flexible residue improves the orientation of H286. Also, QM/MM calculations show that the energy barrier for the aging reaction in this case is very close to reactivation (Lushchekina et al., 2018). More mutations in the acyl-binding loop generated by the combinatorial approach (Terekhov et al., 2017) slightly improved the rate of self-reactivation ($k_3 \sim 0.006\ \text{min}^{-1}$), but calculated energy levels for the reaction pathway show that the intermediate remains unstable (Zlobin et al., 2018a,b).

For comparison, self-reactivating bovine BChE (Dafferner et al., 2017) and swine BChE (Brazzolotto et al., 2015; Nachon et al., 2019) carrying mutations in the acyl-binding loop, but not a new nucleophile pole, have higher rates of reactivation: $k_3 \sim 0.023\ \text{min}^{-1}$ for bovine BChE with chlorpyrifos oxon (the same

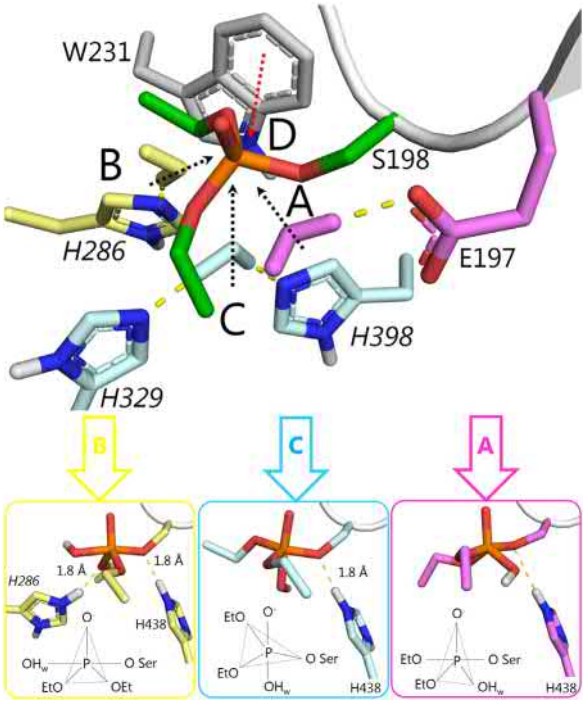

FIGURE 72.9 Possible directions for nucleophilic attack of a water molecule on the phosphorus atom of diethylphosphorylated serine with corresponding configurations of the resulting PI. 2D schemes indicate vertices of trigonal bipyramids. Carbon atoms of the activating histidines and water molecules are colored according to the direction of the attack: direction A, magenta; direction B, yellow, direction C, blue.

phosphylated adduct as for echothiophate) (Dafferner et al., 2017). For swine BChE, only reaction rates with VX have been reported so far (Brazzolotto et al., 2015).

Thus, introduction of a nucleophilic pole in the acyl-binding loop has certain drawbacks: low stability of resulting PI with geometry favorable not only for reactivation, but also for the competing aging reaction.

Another option for nucleophilic attack by a water molecule is direction C (Fig. 72.9). This construct results in PI configuration unfavorable for aging. In this scenario, a pair of mutations, F329H and F398H, suppresses the hydrophobic pocket, creates enough space for a water molecule, and orients the water molecule in a proper position to facilitate hydrolysis of the diethylphospho-adduct. Introduction of two histidines also helps to solve the above-mentioned problem of proton transfer from the PI hydroxyl-group during its dissociation. On other hand, saturation of the active site with histidine residues may result in binding of metal divalent cations, affecting the desired catalytic properties.

Further improvement in the efficiency of nucleophilic attack could be achieved by introduction of amino acids that facilitate protonation of the histidine during activation of the water molecule. This would stabilize the resulting intermediate. Pairs like Glu-Ser, Asp-Ser hydrogen-bonded with histidine mimic the catalytic triad. The role of an acidic residue in such a system is to stabilize the orientation and protonation state of the introduced histidine, increase its pK_a, and cause charge stabilization of an ion pair between the imidazolium cation and the negatively charged PI. Addition of a polar residue like Ser, Thr, Gln, and Asn aims at an additional stabilization of the hydrogen-bonded chain. QM/MM calculations support that such approach is perspective (Lushchekina et al., 2018). However, for that result, multiple mutations are required, what may significantly impair protein stability and lead to partial or complete unfolding. There are reported cases when point mutations led to complete loss of activity (Delacour et al., 2014a,b) due to rearrangement of the hydrogen-bonding network and/or changes in protein dynamics. Changes in the hydrogen-bonding network outside the active side of BChE could reduce conformational stability, but maintain catalytic activity (Grigorenko et al., 2019). This can lead to unexpected catalytic complexities such as slow establishment of steady state (Legler et al., 2014), allosteric effects, and changes in inhibitor sensitivity (Larrimore et al., 2017). Computationally, MD methods, supported by new methods of analysis like principal component analysis and Markov chain models help to explore such issues before making functional expression of proposed mutants.

To summarize, the main approaches to create new BChE-based catalytic bioscavengers are: (1) identification of a geometrically favorable position for a new nucleophile and direction of attack by this nucleophile on the adduct phosphorus; (2) introduction of additional mutations for charge stabilization of intermediates and products; (3) suppression of the competing aging reaction by proper choice of nucleophilic attack direction; and (4) maintenance of overall enzyme stability. The effects of mutations on both the stability and catalytic activity of mutants may be assessed by computational methods: energetic aspects by QM/MM methods, structural and dynamical aspects by MD simulations. However, folding problems that may occur during protein biosynthesis and glycosylation remains an issue to be addressed experimentally after expression.

Then enzyme mutagenesis and expression of computer-designed mutants may lead to new generation of BChE mutants capable of hydrolyzing OPs at high rate. Directed evolution of ChEs could be an alternative to computer-based methods. However, functional expression of ChEs is difficult in yeast, and heavy engineering for bacterial functional expression may lead to misfolded enzymes, thus impairing the desired functionalities of novel ChE mutants.

Bioavailability and biological stability of mutated ChEs for injection is an important issue. The first pharmacokinetic studies of highly purified human BChE injected

to the rat showed that the half-time ($t_{1/2}$) of the enzyme in the bloodstream depends on sialylation of the carbohydrate chains (Douchet et al., 1982). It is well known that rapid elimination of asialoglycoproteins from the circulation is due to their capture by specific receptors located on the surface of hepatocytes. These receptors recognize galactosyl residue, the carbohydrate that precedes sialic acid at the terminus of complex glycans. Studies with other natural and recombinant ChEs confirmed the importance of sialic acid (*N*-acetylneuraminic acid) residues ending glycans (Kronman et al., 1995, 2007; Saxena et al., 1998a,b; Cohen et al., 2007). It was found that $t_{1/2}$ is inversely proportional to the number of unoccupied attachment sites of sialic acid (Kronman et al., 2000). To increase the $t_{1/2}$ of administered recombinant ChEs, all galactosyl residues have to be sialylated. Full sialylation of recombinant enzymes can be achieved using an expression system capable of synthesizing glycans similar to natural human glycoprotein glycans and adding inhibitors of sialidase in the cell culture medium. Coexpression of the enzyme of interest and sialyltransferase in HEK 293 cells was found to lead to fully sialylated recombinant human AChE (Kronman et al., 2000). Alternatively, in vitro sialylation of purified enzymes is possible with a sialyltransferase or using a chemical method (Gregoriadis et al., 1999) and even by cross-linking polysialic acid chains (Ilyushin et al., 2013; Meng et al., 2018). PEGylation has also been proven to be an effective chemical modification for increasing the circulatory half-life of administered recombinant ChE (Cohen et al., 2007; Kronman et al., 2007; Huang et al., 2008). A 150 kDa recombinant fusion protein of human albumin−human BChE showed substantially improved pharmacokinetics when administered to juvenile pigs, $t_{1/2} \approx 32$ h against ≈ 3 h for recombinant 70%-tetrameric BChE (Huang et al., 2008).

Engineering of carboxylesterases to make enzymes capable of hydrolyzing NAs has made progress. In fact, the discovery of a blowfly (*Lucilia cuprina*) resistant to OPs because it carries a mutated carboxylesterase (CaE; LcαE7), G137D, at a position homologous to G117, stimulated research on G117H-based mutants of BChE. Though the OPH activity of the G137D is low, it is balanced by the abundance of the enzyme in the insect organs (Newcomb et al., 1997). The 3D structure of this enzyme was recently solved (Jackson et al., 2013). Knowledge of this structure is a good starting point for engineering of mutants of LcαE7 with improved catalytic activity against OPs. Another interesting CaE is the human CaE1. The 3D structure of human CaE1−NA conjugates (with soman, tabun, sarin, and cyclosarin) was solved (Bencharit et al., 2003; Hemmert et al., 2010). The enzyme was shown to reactivate spontaneously after phosphonylation by the most toxic P_s stereoisomer of sarin (Hemmert et al., 2010). These results guided the computer-modeling design of the first CaE1 mutants displaying OPH activity against soman and cyclosarin (Table 72.1), and enhanced the rate of spontaneous dephosphonylation following sarin inhibition (Hemmert et al., 2011). Thus, CaEs can be reasonably considered as novel catalytic bioscavenger candidates.

72.7 Concluding remarks and future directions

Identified enzymes that neutralize or degrade OPs can be purified from natural sources, for example, human plasma. Recombinant enzymes can be produced using prokaryotic expression systems (*E. coli*), eukaryotic expression systems (yeast, insect, mammalian cell cultures), transgenic animals (worm, rabbit, goat), or transgenic plants (tomato, potato, tobacco, horseradish), and also acellular biosynthetic systems. The goal of current research in protein engineering is to improve mass production of stable and effective muteins at low cost. Regarding OPHs, improvement of in vitro and in vivo catalytic properties toward NAs and pesticides is still the main issue. In addition, improvement of thermodynamic stability (storage stability in solution or in dry forms) and in vivo operational stability, improvement of immunotolerance and bioavailability are other goals. For this purpose, the different strategies of enzyme engineering have been implemented. They consist of research into new natural enzymes, in particular in collections of bacterial strains (Otto et al., 2013) and in extreme environments (Ferrer et al., 2007) or identification of such enzymes from genomic sequences of extremophiles, followed by expression of the synthetic gene in a mesophilic bacterial host, characterization of catalytic properties, and X-ray structure determination (Hiblot et al., 2013a,b). Potential extremozymes, PLL and PROL, have been discovered in halophilic, hyperthermophilic, piezophilic, radioresistant bacteria and archaeas. Research into potential enzymes by computational structure mining in the PDB database is also promising (Jacob et al., 2016). Other enzymes of interest are in insects resistant to OP pesticides, and among secondary targets of OPs in human. Site-directed mutagenesis and directed evolution approaches in combination with chemical modifications and medium manipulations have been used with success to improve the desired properties, in particular stereo-selectivity, high k_{cat}/K_m, and broad spectrum of activity of PTEs (Bershtein and Tawfik, 2008; Goldsmith et al., 2012; Bigley et al., 2013). Computational redesign (molecular modeling and transition state simulations) of known enzymes is another promising strategy. It has already been successfully implemented: the active site of mouse

adenosine deaminase, a zinc enzyme, was redesigned for hydrolysis of OPs. After maturation using directed evolution, the novel enzyme displayed a k_{cat}/K_m activity greater than 10^7-fold the activity of the wild-type enzyme against R_p model OP (Khare et al., 2012). Lastly, pharmacokinetics, toxicokinetics, and immunological studies on animal models are needed to validate the different enzymes of interest.

Catalytic bioscavengers will be part of the arsenal of medical countermeasures for prophylaxis and postexposure treatments of OP poisoning in the very near future. Multiple enzyme associations (enzyme cocktails) will extend the activity spectrum of injected catalytic bioscavengers as well as the efficacy of active components in TSPs, chemical protective clothing suits, and decontamination tools.

Next, gene therapy will offer the possibility of transitory production of human or humanized OP-degrading enzymes in the body. However, the road to gene therapy is still long. In regard to ethical issues, further works are needed to engineer safe vectors that do not produce toxic viral proteins and/or induce immune response. The CRISPR/Cas9 technology offers new possibilities for the development of this approach under safe conditions (Lino et al., 2018; Li et al., 2019).

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Chapter 73

Rapid decontamination of chemical warfare agents from skin

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Abbreviations

AChE	acetylcholinesterase
BChE	butyrylcholinesterase
CWA	chemical warfare agent
DS2	decontamination solution 2
FDA	Food and Drug Administration
GA	tabun, ethyl *N,N*-dimethyl-phosphoramidocyanidate
GB	sarin, isoproppyl-methylphosphonofluroidate
GD	soman, 1,2,2-trimethylpropyl methylphosphonofluoridate
H	impure mustard
HD	distilled sulfur mustard
HTH	high test hypochlorite
L	lewisite
LD_{50}	median lethal dose
RBC	red blood cell
RSDL	Reactive Skin Decontamination Lotion
VX	*o*-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothiolate

73.1 Background: the nature of human skin

Human skin, the largest organ in man, developed as a physical barrier to the environment (keeping things out); on the other hand, it also maintains the aqueous nature of the human body (keeping things in). Mammalian skin consists of three major layers: stratum corneum, epidermis, and dermis. The stratum corneum, the thin outer layer of keratin-filled dead cells (corneocytes) bounded by densely cross-linked protein and embedded in crystalline lamellar lipids, represents the major barrier protecting the body from loss of internal components and entry of undesirable external materials. The epidermis, the layer underneath the stratum corneum, contains cells that change from viable keratinocytes to corneocytes (which are anucleated cells without cytoplasmic organelles) as they migrate from the dermis to the stratum corneum. It also contains a large number of specialized dendritic cells. Smaller amounts of specialized cells are integral to the epidermis, including the pigmentation melanocytes, the immunological Langerhans cells, and the sensory Merkel cells. Throughout the epidermis sebaceous glands, sweat glands, and hair can be found. The next inward layer, the dermis, contains hair follicles with associated sebaceous glands, ecrine sweat glands and ducts, dendritic cells, and a vascular network including subepidermal capillaries, vascular plexi associated with the sweat glands, and dermal papillae associated with the hair follicles. Capillaries are responsible for transporting any chemicals that enter the skin systemically. Recent reviews of the skin structure and permeation are available (Menon, 2002; Hadgraft and Lane, 2005; Godin and Touitou, 2007; Wester and Maibach, 2000).

The stratum corneum, composed of keratinized dead cells that are continually being replaced, is the first major barrier to chemical agents. The barrier qualities of the stratum corneum depend on a number of factors, including its location on the body, which affects thickness and how much hair is present. Thus, hair follicles and sweat glands can either provide channels through the stratum corneum, and thereby bypass its barrier attributes, or at least provide increased surface area for penetration of compounds, since a number of compounds were shown to penetrate faster in hair follicle-rich areas (Illel et al., 1991). Maibach studied three radiolabeled pesticides, parathion, malathion, and carbaryl, for their permeability

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at 13 different anatomical sites in humans (Maibach et al., 1971). Variations in percutaneous penetration were observed; higher penetration of the pesticides occurred at the abdomen and dorsum of the hand.

Because it is often difficult to determine which specific nerve agent was used, most poisoning from pesticides or chemical warfare agents will initially be treated in a general supportive manner and not by administering a specific antidote. Thus decontamination is the most important early intervention (Simpson and Schuman, 2002). Although Maibach et al. (1971) present relatively recent human in vivo studies, most testing of decontamination products is performed either in vivo with animal models (Braue et al., 2011a, b; Clarkson et al., 2012) or in vitro using human skin samples (Mircioiu et al., 2013).

The lipid matrix is another feature important for the barrier function in the epidermis. The arrangement of lamellar-like sheets yields a barrier to hydrophilic compounds and transcutaneous water transport. Extraction of those lipids from skin with organic solvents reduces barrier function (Hadgraft, 2001). The lamellae, which have little phospholipids as they are catabolized, ultimately contain mainly ceramides, cholesterol, and fatty acids (Wertz and Downing, 1989; Bouwstra and Ponec, 2006). The resulting matrix is composed of nonpolar compounds enriched in cholesterol that are adapted to protect the dermis from water loss. While extraction of these lipids may increase the penetration of aqueous moieties, in the case of organophosphates (note the organic-like nature of the chemical warfare agents, as described below), the hydrophobic nature of skin likely facilitates partition of these chemical agents through the lipid matrix, which then enter the subepidermal capillaries for dissemination throughout the body. Further insight into the barrier properties of skin can be observed in disease states including psoriasis, where an increase in epidermal cell replication yields an irregularly stacked stratum corneum and abnormal capillaries in the dermis. This leads to an increase in drug penetration, such as hydrocortisone (Kranz et al., 1977). No studies have evaluated pesticide or chemical warfare agent penetration in psoriasis.

Aging contributes to decreased lipid barrier protection, decreased intercellular cohesion, and increased absorption of toxic material. This barrier is also complicated by environmental effects such as exposure to sun, disease, and other aging processes that include many changes to the structure of the skin. Examples of such changes are decreased amounts of collagen, loss of melanocytes, decreased number of glands and hair follicles, reduced blood flow (Yates and Hiley, 1979), and the loss of lipid content in the stratum corneum (Elias and Ghadially, 2002). Another study found that 11 of 14 pesticides showed different rates of skin penetration in aged rats compared to young rats (Shah et al., 1987). Generally, decreased absorption occurred in studies of aged skin (Fisher et al., 1992; Farage et al., 2007).

Percutaneous absorption in vivo leads to the delivery of the chemical or drug to the microcirculation in the dermis. The period of time that it takes for entrance to the blood supply and circulation throughout the body depends on the diffusion parameters and the interaction with the lipid matrix (Roberts, 1997). Thus, chemicals exhibiting a longer lag time through the skin should be less toxic if quickly removed compared to rapidly penetrating compounds. Another aspect of percutaneous absorption is the number of exposures to the chemical. Some chemicals, such as azone (1-dodecylazacylohepan-2-one), alter the organization of the skin so that an increase in absorption or a synergistic effect is observed with each exposure (Ademola et al., 1993). Chemicals that do not alter the skin's structure would not be likely to increase their bioavailability and absorption, but rather provide an additive response (Bucks et al., 1985).

73.2 Background: nerve agents

Nerve agents are among the most toxic of the known chemical agents. Nerve agents are organophosphates that bind irreversibly to acetylcholinesterase (AChE) (Taylor et al., 1999) and to the bioscavenger butyrylcholinesterase (BChE) (Wolfe et al., 1992) in both the peripheral and central nervous systems. AChE is responsible for terminating the action of the neurotransmitter acetylcholine by hydrolysis. Organophosphate-inhibited AChE results in an excess of acetylcholine and the overstimulation of muscarinic and nicotinic receptors. Characteristic signs of nerve agent poisoning and cholinergic overload include hypersecretion and respiratory distress. When the nerve agent is transported past the blood−brain barrier, convulsions can lead to coma and death. Organophosphates pose a hazard in both their vapor and liquid states. Notably, AChE inhibitors are used not only as a therapy for treating glaucoma, myasthenia gravis, Alzheimer's disease, and atropine poisoning, but also in potentially hazardous ways as pesticides to kill insects and as chemical warfare agents by terrorists and in warfare to kill humans (Sidell, 1997; Leikin et al., 2002; Martin and Lobert, 2003). The use of nerve agents by the Syrian military in 2013 demonstrates that large numbers of fatalities are not only possible but probable when unprepared civilian populations are attacked (Chai et al., 2017).

The nerve agents include the G-type agents GA (tabun, ethyl *N,N*-dimethyl-phosphoramidocyanidate), GB (sarin, isoproppyl-methylphosphonofluroidate), and GD (soman, 1,2,2-trimethylpropyl methylphosphonofluoridate), and V-type agents such as VX (*o*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothiolate). The V-type nerve agents are several orders of magnitude less

volatile than the G-type agents and act primarily as a liquid via the percutaneous route; for example, VX is several orders of magnitude more lethal percutaneously than GB (Reutter, 1999; Braue et al., 2011a) or GD (Clarkson et al., 2012; Braue et al., 2011b).

Log *P* data (octanol:water partition coefficients and a reflection of lipid solubility) of nerve agents were used to both predict absorption through the skin and determine the distribution of organophosphate compounds in tissues, and were then correlated with toxicity as measured by the onset time of fasciculation in guinea pigs. An excellent correlation ($r = 0.95$) was established between the measured log *P* value and the time local fasciculations were visually observed (Czerwinski et al., 2006). The authors suggest the log *P* data can be used to estimate absorption in the skin, penetration to blood, and dissemination to muscle tissue throughout the animal (Czerwinski et al., 2006).

Maxwell and Lenz reported that AChE is more reactive with cationic nerve agents such as VX when compared to neutral agents that contain less than two bulky groups (e.g., GA, GB, and GD) (Maxwell and Lenz, 1992). Investigations by Tammelin revealed that organophosphates containing quaternary choline-like side groups were about 1000 times more reactive with AChE than their tertiary counterparts (Tammelin, 1958). In general, since AChE has a smaller active site than BChE, the size and ionic character of the active sites determine the specificity of these esterases for the agents. The estimated ranked percutaneous nerve agent LD_{50}s are VX > GD > GA > GB, which corresponds to their volatility. The ranked volatility for these agents is VX < GA < GD < GB.

Using parathion as a model simulant for the nerve agent VX, the in vitro percutaneous absorption through unprotected human skin and clothed and uniformed skin was determined. The percent parathion dose absorbed through the unprotected skin was significantly greater than that observed through dry uniformed skin, while absorption was higher through the wet (sweat) uniform. These results suggested that military uniforms and clothing worn in public places provide protection to this simulant and by analogy to VX; but absorption through cloth and skin quantitatively occurred more readily with wet clothing than dry. Thus, even with clothing, immediate responses and decontamination of skin and clothing are required (Wester et al., 2000).

In conclusion, because of the extreme toxicity of nerve agents, the search for medical decontamination countermeasures to organophosphates is of paramount importance. Two recent assassination attempts, the successful assassination of Kim Jong-Nam (Chai et al., 2017) and the unsuccessful assassination attempt of Sergei Skripal and his daughter, Yulia (Chai et al., 2018), have increased interest in the study of dermal absorption of nerve agents and demonstrated the need for effective decontamination. Rapid removal from the skin would prevent penetration to the general circulation and the resulting decrements of cholinergic toxicity, which ultimately leads to seizure and/or death in untreated individuals. In the development of medical decontamination countermeasures to nerve agent poisoning, different nerve agent administration routes are acknowledged to likely have different requirements for effective treatment. The window of opportunity for decontamination treatment following agent exposure is limited (Joosen et al., 2017). The signs of poisoning develop within minutes, and if decontamination is delayed, toxic levels of the nerve agents are likely to be disseminated via the bloodstream after the agent has been absorbed. Decontamination will prevent continued absorption of the agent, reducing the need for further medical management (Table 73.1) (Hamilton et al., 2004; Clarkson et al., 2004, 2012).

TABLE 73.1 VX applied to pig skin (ear).

Decontamination	
Delay (min)	**Signs**
0	–
15 (no decontamination)	+++
15 (decontamination)	+

Data from Hamilton et al., 2004

73.3 Background: vesicating agents (distilled sulfur mustard, HD; impure sulfur mustard, H; Lewisite, L)

Sulfur mustard (HD), a synthetic vesicating agent, was a major chemical warfare agent during World War I and continues to be a modern-day threat (Reutter, 1999; Ghanei and Harandi, 2007; Bismuth et al, 2004; Sezigen et al, 2019). Sulfur mustard's simple and cheap chemical synthesis makes it readily accessible to terrorists and for use by the military. Sulfur mustard is an alkylating agent that causes its damage by disrupting nucleic acids and proteins, impairing cell homeostasis and eventually causing cell death, although the significance of the multiple pathways is unclear (Smith et al., 1995). Whole-body exposure results in cutaneous (liquefaction necrosis of the epidermis), respiratory (injury to the laryngeal and tracheobronchial mucosa), and ocular effects (severe conjunctivitis). In contrast to HD agents, there is no delay

with lewisite (L), which produces immediate burning of the skin and eyes. Compared with the G-nerve agents, sulfur mustard has a relatively low acute lethal toxicity; that is, its toxicity as an incapacitating agent is of much greater concern than its capacity to kill. Furthermore, HD is persistent in the soil and other materials for hours to weeks (Devereaux et al., 2002).

The skin is an important port of entry for vesicating agents. The agent's lipophilic nature and the propensity of skin to exclude aqueous compounds but not lipophilic substances make the skin an unwitting transport system. An increase in ambient temperature causes increased penetration (which was used effectively in World War I, where mustard was mostly disseminated at night and warmed in the early morning sun). It has been estimated that 80% of liquid mustard evaporates and 20% penetrates the skin. Of this 20%, 12% is retained in the skin matrix, while 8% is absorbed systemically, so only large dosages of mustard will produce significant systemic toxicities (Cullumbine, 1947; Dacre and Goldman, 1996).

Mustard skin lesions first present as erythema followed by blisters (Somani and Babu, 1989). Erythema usually begins 2–24 h after contact, followed by acute itching, which diminishes as the characteristic blisters appear. Blisters initially appear as small vesicles within the area of erythema 18 h after contamination; these vesicles then coalesce to form the characteristic pendulous blisters containing large volumes of clear but yellow fluid. Blisters are not painful per se, but they may be uncomfortable and may feel tense. Warm, moist areas such as genitalia and axilla are more likely to exhibit bullous lesions. By 48 h postexposure, blistering is clearly evident and a new round of blisters appears. As the skin layer is disrupted, the large blisters break, leading to erosions and full-thickness skin loss (in which subcutaneous fat may be visible but bone, tendon, or muscle are not exposed), and ulceration, necrosis and, 72 h postexposure, formation of an eschar. The eschar sloughs in a 4- to 6-day time period, finally leaving a pigmented scar (Reid et al., 2000, 2007). The burn caused by blister agents is much slower to heal in comparison with a thermal burn, likely because of the multiple mechanisms by which the agent affects biological tissue, as known from World War I and reestablished in Iranian casualties from the Iran–Iraq War. The site of healed mustard burns is hypersensitive to mechanical trauma. In a comparison of cutaneous lesions in 500 mustard-exposed Iranian veterans and 500 unexposed veterans, a correlation was observed between exposure and skin lesions and severe dry skin, hyper- and hypopigmentation, local hair loss, eczema, and chronic urticaria. Histopathological examination of skin biopsies has revealed nonspecific findings, including epidermal atrophy, keratosis, and basal membrane hyperpigmentation (Balali-Mood and Hefanzi, 2006).

73.4 Model systems to measure absorption, removal, and decontamination

73.4.1 Rats

Many different animal models have been used to assess the percutaneous absorption of toxic chemicals. There is little question that while in vivo human studies are best for predicting the absorption of percutaneously applied chemical warfare agents, ethics preclude conducting such studies. Rats have been widely used in the study of skin contamination, wounds, and healing and the efficacy of different decontamination modalities (Wester and Maibach, 2000; Shah et al., 1987; Baynes et al., 1997).

73.4.2 Guinea pigs

While rats are often selected for their availability, low cost, small size, and thorough biological characterization, rats are not the ideal chemical warfare agent model because they contain a high amount of carboxylesterase, a potential hydrolytic enzyme for organophosphates (Sweeney and Maxwell, 2003). Unlike rats, humans have small amounts of this enzyme relative to AChE and BChE. To overcome this limitation, the guinea pig, which exhibits low carboxylesterase, has been developed as a model for chemical warfare agent exposure (Fonnum et al., 1985). Guinea pigs have been evaluated for skin damage from burns and are often used as a wound-healing model for sulfur mustard (Ramos et al., 2008), as well as for skin irritation to toxic industrial chemicals (Kennedy, 2007; Weaver et al., 2003). Guinea pigs have also been used to study absorption of chemical warfare agents through the skin (Dalton et al., 2006; Wormser et al., 2002) and uptake of radioactive sulfur mustard through the skin (Logan et al., 1999), as well as an animal model for evaluating pretreatment regimens to protect against chemical warfare agents (Wetherell et al., 2006) and for assessing cholinesterase activity responses (Haigh et al., 2005) to GD exposure and organophosphate-induced seizures (Harrison et al., 2004).

For evaluating the decontamination of guinea pig skin, Clarkson et al. (2012) sedated and clipped guinea pigs which were then cutaneously exposed to neat nerve agents on their sides. Animals had their fur removed using Oster brand clippers (model: Golden A5) with a #4 blade CryogenX blade instead of being shaved with shaving cream and a razor blade, as previous work showed that shaving caused razor burn and increased the rate and amount of agent absorbed. One minute after the exposure, a sponge wrapped around a pair of forceps was moved across the guinea pig's side; then the forceps were rotated 180 degrees so that the clean surface of the sponge was

pointed at the animal. Three more passes were taken from the rear toward the front. An identical procedure was used when the protocol required an additional second sponge to decontaminate the animal. Similarly, guinea pigs were used for studies on the decontamination of sulfur mustard. In this case, 24 h after neat HD exposure and decontamination, animals were injected with trypan blue and then euthanized. The skin covering the backs of the animals was removed. In addition, skin punches were taken from each of the exposure sites (control, exposed, and decontaminated sites) (Gordon et al., 1999; Gordon and Doctor, 2003).

73.4.3 Swine

Pig skin has long been a valuable model for human skin (Meyer et al., 1978; Riviere and Monteiro-Riviere, 1991) as it expresses a sparse hair covering, epidermis, and an arrangement of dermal collagen and elastic fibers similar to that of human skin. Again, many investigations have used the porcine skin model to study cutaneous toxicology of HD (Gold et al., 1994). Pig skin, because of its similarity to human skin, with respect to hair covering, apocrine sweat glands, and other morphological similarities (Reifenrath et al., 1991), is an attractive model for cutaneous absorption and toxicology studies of organophosphate chemical warfare agents (Hamilton et al., 2004). Cutaneous absorption studies show that pig skin permeability, compared to that of the rat and rabbit, most closely resembles that of human skin (Bartek et al., 1972) with a variety of test agents. Because porcine skin can be easily mounted on static skin diffusion cells and the penetration of radiolabeled agent quantified, this model is currently widely in use (Matar et al., 2019). Therefore the pig is a good model in which to assess the effects of extraneous material or chemicals during the early events of exposure. The downside is that pigs are large animals, are difficult to house, are more costly, and require special cages to maintain them in comparison to rodents. Their size creates a serious caging issue when neat chemical warfare agents are applied to skin, because the animals are required to remain in the chemical fume hood until the agent has off gassed or been neutralized by decontamination. This process can take several days, which creates not only logistical problems but also animal welfare problems since strict guidelines govern how long an animal can be kept in a cage small enough to fit in the hood.

73.5 Decontamination requirements

Medical decontamination requires removal and/or neutralization of chemical warfare agents, which upon penetration of the skin produce vesication or, with organophosphates, enter the systemic circulation and poison cholinesterases. The most important process for the exposed soldier or civilian is to remove the chemical agent from the skin as quickly as possible. The soldier, under harsh conditions, must use the product quickly to minimize transdermal penetration. A decontaminant that inactivates the chemical agent prevents its penetration through the skin and potentially protects a medical worker or buddy from suffering a second-hand exposure.

Another criterion for the decontaminating system and reagents is that it be as universal as possible and protect against the various classes of chemical agents (as well as radiochemicals and biological agents, although the latter compounds are not discussed here). In other words, the soldier has a limited amount of space and weight to carry, and cannot carry multiple decontamination schemes. Furthermore, a soldier is unlikely to be able to determine the type of agent with which he is contaminated in the absence of symptoms.

In addition, proven efficacy of a decontamination product would have to meet Food and Drug Administration (FDA) guidelines and approval, assuring the safety of the product for the soldier. The product should be environmentally safe to use by itself and render the chemical/biological agent environmentally safe, to prevent cross-contamination. Logistics would preclude decontamination products that require freezing or refrigeration, since they would not be available in the field. Last, application of the product needs to be simple so that the soldier can easily use the product under stressful conditions, thereby reducing the probability of failure. Products like the M291 Skin Decontamination Kit (SDK) (see below) are used by simply wiping the contaminated skin and do not require significant training. However, the M291 SDK has some drawbacks, such as a black, irritating dust that must not be allowed to contact the eye. The ideal decontaminating product should be nonirritating and nonallergenic and should not have an offensive odor (as some potential mercapto compounds exhibit) (Shi et al., 2008); otherwise, the product will be hesitantly used.

Methods for decontamination, neutralization, and removal of chemicals, such as organophosphorus and organosulfur compounds, herbicides, and insecticides, are discussed in the literature (Hurst, 1997; Houston and Hendrickson, 2005; Rosenberg, 2005; Baker, 2004). The compositions and devices utilized for medical purposes are markedly different from nonmedical devices; the latter are not compatible with the skin or other sensitive tissues because they can be corrosive, flammable, toxic, difficult to make and store, composed of two-component systems, and limited in their shelf-life. For example, DS2, a standard decontamination agent, is comprised of 70% diethylenetriamine, 28% ethylene glycol monomethyl ether, and 2% NaOH by weight (Modec, 2003). Although DS2 is effective, it is corrosive upon exposure to air. DS2 as well

as any matter resulting from its use is classified and regulated as hazardous material. After an application, the DS2 must stand for 30 min before rinsing the treated area with water. Additionally, DS2 comprises a teratogen. Clearly, this is not a better method for neutralizing, detoxifying, decontaminating, and cleaning personnel exposed to chemical warfare compounds.

73.6 Decontamination schemes

Organophosphorus nerve agents are a serious threat to military and civilian personnel, but not just from exposure during warfare or a terrorist event; with these agents the possibility exists that exposed individuals could cross-contaminate the medical personnel treating them. Several medical decontamination schemes, each exhibiting advantages and disadvantages, are described below. While the ideal candidate does not exist, the final product must be field deployable for the individual—that is, for personal use in a rapid, deployable manner. Simple materials such as bleach and complex products such as organophosphate-degrading immobilized enzymes sponges are described. Inexpensive and readily available household materials were tested for pesticide decontamination of fabric materials over 20 years ago (Easter and DeJonge, 1985). The household products provided only marginal decontamination efficacy.

73.6.1 Classical liquid: sodium hypochlorite (bleach)

Decontamination methods employing hypochlorite formulations have some corrosive and toxic side effects. A Chlorox (hypochlorite) solution is composed of household bleach, which is about 5% sodium hypochlorite. Thus, for a 0.5% solution, bleach is mixed with nine parts water, although even this diluted solution is contraindicated for use in or on a number of anatomical areas including the eye.

Undiluted bleach is not used because it is toxic to the skin and may create more damage than no decontaminant. Hairless guinea pigs were exposed to sulfur mustard in wounds and the surrounding intact skin, and then decontaminated with water, 0.5%, or 2.5% bleach. No significant differences were observed among wounds decontaminated with the three solutions. Unexpectedly, the skin surrounding nondecontaminated (but exposed) control animals showed the least visual pathology. The lesions observed after decontamination might be due to the mechanical flushing of sulfur mustard onto the perilesional skin, by chemical damage of the skin induced by the solution, enhanced penetration of the agent, or interaction of sulfur mustard with the decontaminating solutions (Gold et al., 1994). In a study evaluating decontamination of GB from skin, rabbits that received GB but were not decontaminated were observed to neither convulse nor die. In contrast, when decontaminated with 5% bleach, symptoms and death increased, suggesting that 5% bleach perturbed the protective barrier of the skin or facilitated GB transport through the skin (Kondritzer et al., 1959). Since diluted bleach (0.5%) is a nonirritant to human skin, it is preferred (Racioppi et al., 1994).

In contrast, the effectiveness of diluted bleach has been demonstrated. This study measured the rate of sulfur mustard disappearance from the skin after topical application of the vesicant, which rapidly penetrates the skin because of its hydrophobicity. Three swabbing treatments of undiluted HD-exposed skin with gauze pads soaked in 0.5% hypochlorite caused a 68% reduction in skin HD content and a 64% reduction when hypochlorite was replaced by water (Wormser et al., 2002). The effectiveness of 0.5% hypochlorite with water for decontaminating sulfur mustard on guinea pigs was also evaluated. However, the gauze pads soaked with the bleach contained microgram quantities of HD when water was used but no detectable HD levels when 0.5% bleach was used. Thus, the neutralizing effect of 0.5% bleach occurred after the agent was removed from the skin and away from the lipophilic structures of the skin where the 0.5% hypochlorite could react with and reduce the levels of the agent.

Similar to bleach's oxidative iodine properties, topical povidone-iodine at 15 and 30 min postexposure to sulfur mustard exhibited protective effects. Severity of the dermal parameters, acute inflammation, and dermal necrosis were significantly reduced, and reduced skin damage was observed in areas adjacent to treated sites (Brodsky and Wormser, 2007).

The impact of this work using 0.5% bleach has been significant (Wormser et al., 2002). US Navy warships are equipped to operate in a chemical, biological, or radiological warfare environment where their exterior surfaces may become contaminated while the interior is kept clean. Most of these ships have decontamination facilities accessible from the ship's deck. Thus, in a contaminated environment, patients would be brought to the ship's facilities by personnel wearing protective gear. Next, contaminated clothing would be removed and contaminated skin washed with 0.5% bleach. Because of space considerations this 0.5% bleach solution is made by dissolving High Test Hypochlorite (HTH). HTH is a dangerous corrosive powder that contains a chlorine concentration of 65%−70%. After washing with 0.5% bleach, the patients are rinsed with warm water and monitored to determine if it is safe to bring the decontaminated personnel into the interior of the ship for treatment (National Academy of Sciences, 2004).

73.6.2 Powder decontamination material: M291 SDK (Fig. 73.1)

In the 1990s, the product provided to the US soldier for field use was the M291 SDK. For 15 years, the US military fielded the M291 SDK, but in the late 2000s the US began to replace it with the Reactive Skin Decontamination Lotion (RSDL). However, throughout the world, many decontamination facilities continue to use the M291 SDK, and therefore it is important to discuss it in detail here. In the M291 SDK three main components are incorporated into individual pouches: a fiber pad (six to a pouch), an absorbent activated charcoal, and a reactive resin (Ambergard XE-555). Each component serves a unique purpose. First, the cotton pad provides structural integrity for use on a finger. Second, the carbon incorporated into the pad absorbs organic material such as organophosphates and HD. Third, the ion-exchange resin binds chemical agents and very slowly detoxifies them. The soldier takes the M291 SDK pad and rubs the area that needs to be decontaminated. The goal is to rapidly bind chemical agents still on the surface of the skin and prevent their penetration through the stratum corneum. The M291 SDK is precluded from use in the eye because its particulate nature is irritating, but it can otherwise be used on the face and around wounds. Much of the black powder from the M291 SDK remains on the skin, which has been a deterrent to its use. The presence of the black powder is an indication to the soldier that the area has been decontaminated. However, since ion-exchange resin binds chemical agents and very slowly detoxifies them, for maximum effectiveness it is best to brush off excess black powder as this powder can contain agent that has not been inactivated.

Efficacy of the M291 SDK has been evaluated in a number of animal models for organophosphate poisoning (DO49 Technical Report, 1987). In an early report, rabbit skin was shaved (to mimic human skin without fur) and then the skin exposed to GD and VX for 2 min. Decontamination with the M291 SDK yielded higher LD_{50}s [of more than 10- and 20-fold, respectively (Hobson et al., 1985)], in comparison to control (nondecontaminated) animals. In another study, with rabbits under similar conditions, the penetration of the organophosphate VX was measured by red blood cell AChE inhibition. The M291 SDK increased the amount of VX required to inhibit AChE by 50% (Joiner et al., 1988). Thus, the M291 SDK neutralized and/or removed VX so that less penetrated through the skin to be systemically delivered as indicated by RBC AChE inhibition.

Three recent reports examined the effectiveness of the M291 SDK in treating GD and VX, using an animal model of anesthetized clipped-hair guinea pigs (Braue et al., 2011a,b; Clarkson et al., 2012); the LD_{50}s were determined at 24 h. Clarkson et al. (2012) reported that decontamination with the M291 SDK 1 min after neat (undiluted) exposure to GD increased the LD_{50} from 11.6 to 76.9 mg/kg, yielding a protective ratio of 6.6. Decontamination with the M291 SDK 1 min after neat exposure to VX increased the LD_{50} from 0.10 to 0.87 mg/kg, yielding a protective ratio of 8.7. In contrast, Braue et al. (2011a,b) reported that decontamination with the M291 SDK 2 min after neat exposure to GD increased the LD_{50} from 11.0 to 30.0 mg/kg, yielding a protective ratio of 2.7, and decontamination after neat exposure to VX increased the LD_{50} from 0.13 to 0.14 mg/kg, yielding a protective ratio of 1.1. The untreated LD_{50}s in the studies discussed here were similar, while the difference in protective ratio for GD can be explained by the time difference in decontamination (1 vs 2 min). GD penetrates skin faster, so the additional minute delay can have a significant impact on dissemination of the nerve agent systematically compared to the slower penetrating VX, which is reflected in the decreased protective ratio. The difference in protective ratios for VX is more difficult to explain, as VX does not penetrate to the systemic circulation as rapidly as GD.

The M291 SDK is also efficacious in rabbits against the vesicating agents HD and L. The shaved dorsal skin of rabbits was exposed to neat HD and then decontaminated after 1 min. Vesicant-induced histopathology determined after tissue staining was reduced over 20-fold compared to nondecontaminated control rabbits.

FIGURE 73.1 M291 SDK (www.defenselink.mil).

73.6.3 Liquid decontamination material: Sandia foam (Fig. 73.2)

Sandia National Laboratories decontaminating foam (licensed to Modec Inc., Denver, Colorado) is a solution (MDF-100) composed of two parts: part a is a solution of 6.6% *N,N,N,N′,N′*-penta-methyl,-*N′*-tallow alkyl 1,3-propanamine diammonium, 2.6% tallow pentamethyl propane quaternary ammonium compounds, benzyl-C12-18 alkyl dimethyl, and 1% isopropyl alcohol; part b is a solution of 8% hydrogen peroxide. Mixing the two parts results in a foam-like product which lasts for up to 30 min. The mixture was tested in the guinea pig model, where fur was clipped on the side of the animal 1 day before exposing the skin of the anesthetized animals to neat GD or VX (Lukey et al., 2004; Clarkson et al., 2012). The animals were decontaminated 1 min later with sterile gauze soaked in the combined solution in a defined manner: the contaminated side was wiped across the exposure site in the direction of the shaved fur once and then rotated so that a clean surface of the gauze could be used to wipe the skin for three additional passes. Next, the organophosphate-exposed area was similarly dried with a second piece of gauze. The exposed area was wiped a total of eight times. Twenty-four hours later, survival was determined. Control nondecontaminated animals yielded an LD_{50} of 11.6 mg/kg for GD, while animals decontaminated with the mixture yielded an LD_{50} of 412 mg/kg, a 36-fold protective ratio. For VX, cutaneous neat exposure and decontamination with Sandia foam yielded an LD_{50} of 10.4 mg/kg compared to the control animals' LD_{50} of 0.10 mg/kg, a 104-fold protective ratio.

FIGURE 73.2 Sandia foam.

Despite its efficacy, Sandia foam has a number of drawbacks for field use by the soldier. First, it must be stored as separate components, which would require a rapid, personal, on-site mixing chamber for combining the two solutions. Second, the presence of hydrogen peroxide, a strong oxidizing agent, precludes its use near the eye, and would create much discomfort if used in a wound (Watt et al., 2004). To partially address these concerns, Sandia developed the formulation DF-200, which in part, contains less hydrogen peroxide and surfactant.

73.6.4 Liquid decontamination material: Diphotérine

Diphotérine is a product for chemical spatters on the eye and skin. Prevor Laboratory in France manufactures this odorless, colorless liquid dispensed as an eye wash or skin decontamination spray. It is composed of an aqueous solution to wash many chemical families and pull hydrophilic chemical agents away from the surface of tissues, an amphoteric solution that acts on acids and bases and restores the tissue physiological pH, and a hypertonic solution that stops penetration of corrosive chemicals into tissues. The pH is slightly alkaline (pH 7.2−7.7) and sterile. Although not classified as a medical device in the United States, it is classified as such in Europe, Canada, Australia, and Brazil (www.prevor.com).

Diphotérine's action on more than 600 chemical compounds was reviewed (Hall et al., 2002). These chemicals included acids, alkalis, oxidizing and reducing agents, irritants, lacrimators, solvents, alkylating agents, and radionuclides. In the literature, there is one abstract describing the decontamination of sulfur mustard (Gerasimo et al., 2000). In this report, radiolabeled sulfur mustard was placed on human skin for 5 min in vitro. The skin was then treated with Diphotérine, water and soap, or saline at different time periods after sulfur mustard exposure, and Diphotérine was reported to be significantly better at removing sulfur mustard. No reports could be found in the literature for organophosphate decontamination, although Hall et al. (2002) also state that Diphotérine is suitable for decontamination of organophosphate pesticides.

Evaluation of Diphotérine has occurred mainly in the European workplace, where it has been reported to be nonirritating to normal human eyes or skin. No adverse effects have been observed in ongoing postmarketing surveillance in European industrial facilities (Hall et al., 2009). Diphotérine is nontoxic in guinea pigs and does not sensitize their skin (Mathieu et al., 2007). The product has prevented or decreased the severity of chemical eye/skin burns from 96% sulfuric acid, 100% acrylic acid, 50% acrylamide, solid sodium hydroxide flakes, and

dimethylethylamine; 24 workers in a German metallurgy facility who were exposed to weak or strong acids and bases and obtained immediate Diphotérine decontamination did not require further medical or surgical burn treatment nor did eye/skin burns develop (Nehles et al., 2006). Clearly, Diphotérine's potential is intriguing and needs to be critically evaluated for decontamination and detoxification of chemical warfare agents.

73.6.5 Liquid and sponges: Reactive Skin Decontamination Lotion (Fig. 73.3)

RSDL was developed for cutaneous decontamination of chemical warfare agents. It was developed for topical use by the Defense Research Establishment in Suffield, Canada, with broad-spectrum decontamination properties for chemical agent cutaneous threats. The RSDL solution is composed of 1.25 M potassium 2,3-butanedione monoximate in polyethylene glycol monoethyl ethers of average molecular weight 550 daltons ($MPEG_{550}$) with 10% w/w water (pH 10.6). The pads consist of a sponge-like plastic foam, Opcell, which is lightweight and easy to store. It is used instead of a cotton pad, and the Opcell holds more of the decontaminating solution for spreading on the skin.

The toxicological profile of this formulation was determined by the US military prior to FDA (Food and Drug Administration) clearance (Tonucci et al., 2004). The Army tested the product's safety by conducting skin irritation, sensitization, and photo-irritation studies in more than 300 people. Its effectiveness was also tested by treating animals that had been exposed to chemical agents. On March 28, 2003, the product was approved by the FDA to remove or neutralize chemical warfare agents and T-2 fungal toxin from the skin (but not other biological threat agents or radiological contaminants).

The efficacy of RSDL has been demonstrated. RSDL was selected as the Joint Service (Army, Navy and Air Force) Personnel Decontamination System (JSPDS) in March 2007. It is also an approved medical device for nerve agent decontamination in the EU, Australia, and Canada. The efficacy of RSDL to remove and decontaminate chemical warfare agents was demonstrated in vivo with guinea pigs and in vitro with chick embryos.

The in vitro work by Sawyer et al. (1991) involved primary cultures of chick embryo neurons to test the efficacy of the RSDL. By relating the anticholinesterase activity in these cultures of the organophosphate/RSDL mixture to that of pure organophosphate standards, a sensitive measure of the value of the RSDL in inactivating tabun, sarin, soman, and VX was obtained. Data from experiments with all four nerve agents in this in vitro system correlated well with the in vivo data, and also indicated that the inactivation process was time- and agent-dependent and was related to the ratio of organophosphate to RSDL. The authors showed that RSDL is also effective in decontaminating GD and VX in cutaneously exposed guinea pigs (Table 73.2; Gordon, unpublished observations). The protective ratios reported in Table 73.2 are in agreement with the protective ratios reported by Braue et al. (2011a,b).

The product was also compared to Fuller's earth in a pig model. The potency of the RSDL/sponge was statistically better than Fuller's earth against skin injury induced by sulfur mustard, observed 3 days postexposure. RSDL was more efficient than Fuller's earth in reducing the formation of perinuclear vacuoles and inflammation processes in the epidermis and dermis. The potencies of the RSDL/sponge and Fuller's earth were similar to severe inhibition of plasma cholinesterases induced by VX poisoning. Both systems completely prevented cholinesterase inhibition, which indirectly indicates a prevention of toxic absorption through the skin (Taysse et al., 2007).

FIGURE 73.3 Reactive Skin Decontamination Lotion (RSDL; www.nbcdefence.net).

However, there are some caveats for the use of RSDL. The application of RSDL directly to open wounds impaired wound strength and decreased collagen content in the early phases of wound healing. This may have clinical implications for the treatment and outcomes of chemical casualty combat trauma (Walters et al., 2007). RSDL is also reported to be flammable.

73.6.6 Polyurethane sponge (Fig. 73.4)

At the Walter Reed Army Institute of Research, an enzyme-immobilized polyurethane foam sponge to decontaminate the skin of chemical warfare agents in a wide variety of environmental conditions is being developed (Munnecke, 1979; Wood et al., 1982; Havens and Rase, 1993). A porous polyurethane foam formed in situ from water-miscible hydrophilic urethane prepolymers has been combined with enzymes such as ChEs to produce immobilized enzyme sponges (Gordon et al., 1999; Gordon and Doctor, 2003; Ember, 1997; Medlin, 1998). In this method, the enzyme becomes an integral part of the solid support. Some of the advantages of this technique include retention of similar kinetic characteristics as the soluble form of the enzyme. Most important, the immobilized enzyme retains high activity after prolonged storage and is resistant to the detrimental effects of low and high temperatures and to long exposure to the environment. In addition, the enzymes are covalently attached to the polyurethane, so they will not leach from this polymer support.

To increase the organophosphate/enzyme stoichiometry, polyurethane-immobilized enzymes were combined with oximes (enzyme reactivators such as HI-6; Peter et al., 2007) so that the catalytic activity of organophosphate-inhibited AChE (or BChE) is rapidly and continuously restored before irreversible aging of the enzyme−organophosphate complex can occur. Thus, the organophosphate is continuously detoxified. A reusable immobilized-enzyme sponge of cholinesterases and oximes for organophosphate decontamination is the envisioned product. Next, it was demonstrated that the organophosphates diisopropylfluoro-phosphate (DPF) or MEPQ [7-(methyl-ethoxyphosphinyloxy)-1-methylquinolinium iodide] inhibited the activity of ChE-sponges, as was observed for nonimmobilized ChE in solution. The oxime HI-6 restored activity of the AChE-sponge until the molar concentration of MEPQ reached approximately 1000 times that of the cholinesterase active site. However, the AChE-sponge could be recycled many times by rinsing the sponge with HI-6 in the absence of OP. In this case, most of the original ChE activity would be restored to the sponge. Therefore, the bioscavenger approach could be used externally: the sponge would soak up organophosphate, decontaminating the contaminated skin (Caranto et al., 1994). Then the ChE sponge and oxime would detoxify the OP in the sponge. We found that the ability of the immobilized enzymes and HI-6 to detoxify the organophosphate MEPQ was dependent upon the efficiency of the sponge to decontaminate particular surfaces including plastic and guinea pig skin.

Characteristics of polyurethane immobilized enzymes: The longevity of sponges composed of immobilized cholinesterases is greater than 5 years at room temperature (not shown). The immobilized enzymes are also very stable in aqueous environments. One significant

TABLE 73.2 Decontamination of organophosphate-exposed guinea pigs.

Agent/treatment	LD_{50} (mg/kg)	PR[a]
Soman		
M291 SDK	17.7	1.8
RSDL	240	24
Sponge	290	29
None	9.9	n/a
VX		
M291 SDK	0.14	n/a
RSDL	19.1	137
Sponge	21.0	150
none	0.14	n/a

[a]*Protective ratio.*

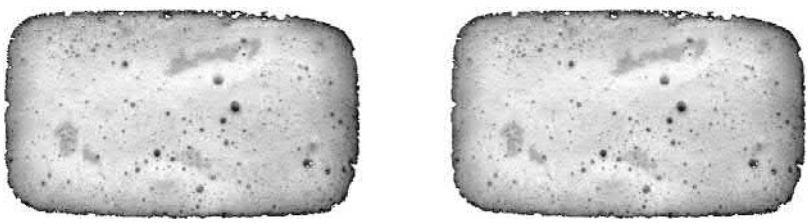

FIGURE 73.4 Polyurethane sponge.

difference and advantage that the immobilized enzymes have compared to the soluble cholinesterases is that immobilized enzymes do not dissociate (leach) from the sponge. Therefore, the immobilized enzymes can be left in the liquid or other environments. For instance, the AChE activity in the immobilized sponge was stable for more than 60 days in continuous immersion in aqueous samples including Allegheny River fresh water or brackish water (Gordon et al., 2002). Since the results were identical for autoclaved and untreated water, the immobilized enzymes were also resistant to microbial-induced proteolytic degradation. Also, note that the same sponge was assayed multiple times over many days, so it is evident that the immobilization process confers dramatic stability to covalently coupled cholinesterases and that the enzymes do not leach from the polyurethane matrix.

Organophosphate removal by the sponge and formulation: The capacity of sponges to remove GD or organophosphates from guinea pig skin was determined using a back-titration method, where removed organophosphate in the sponge was added to a known amount of cholinesterase. Inhibition of the exogenously added cholinesterase permits quantitation of the organophosphate concentration. From these studies, it was determined that additional components when added to the sponge improved the sponge's efficacy by leaching out the chemical warfare agents. In this case, we settled on tetraglyme, which has a propensity to dissolve organic-like materials, including organophosphates (and sulfur mustard, see below). In part, the inability of sponge-tetraglyme to remove all the GD likely reflects the rapid penetration of GD through skin and the inability of tetraglyme to extract this fraction. It also points to the requirement for as rapid a decontamination as possible. These results clearly demonstrate that the sponge not only removed organophosphate from the skin of the guinea pigs, but also in the presence of oxime effectively and completely detoxified the organophosphates within hours. Thus, these sponges would not pose any additional cross-contamination hazard.

Protective ratios of the sponge: It proved to be impossible to modify the prepolymer since currently there is no formulation with an increased hydrophobic nature that might be expected to absorb the organophosphates more effectively. Instead, we utilized additives described above to provide the additional ability to remove soman from the skin, protecting guinea pigs significantly better than the M291 SDK (Table 73.2). A comparison with RSDL is also shown. Compared to the LD_{50} values of 9.9 and 17.7 mg/kg for untreated animals (nondecontaminated) and the M291 SDK, respectively, the sponge provided an LD_{50} of 290. This combination is also effective against VX-contaminated guinea pigs: the sponge increased the LD_{50} from 0.14 to 21 mg/kg, yielding a protective ratio of 150. RSDL provided a protective ratio of 150.

Sulfur mustard decontamination and formulation: The sponge was used to wipe guinea pig skin contaminated with neat sulfur mustard. The following day, the animals were injected with trypan blue. Those areas representing vesicant injury take up the dye. It was observed that the neat HD-exposed tissue (positive control) had a significant dye uptake, while the area decontaminated by the sponge had only a slight uptake of the dye. The control had no dye uptake. In addition, the amount of HD taken up and removed by the sponge was measured over time. While neat mustard remained after 15 min in water, the corresponding amounts were destroyed in the matrix of the sponge and additives. Histopathology of the HD-exposed skin specimens after 24 h demonstrated microvesicles, coagulation at the dermal interface, and in the most severe cases, dermal coagulation. Overall, HD-exposed areas decontaminated with the sponge exhibited characteristics associated with reduced exposure (microvesicles). Thus, these sponges could reduce the damage that HD produces. Cholinesterases may provide sinks as alkylation sites for the sulfur mustard and account for some of the reduced toxicity of the agent upon sponge decontamination. Another feature demonstrated was that the tetraglyme leaches from skin not only organophosphates, but also the organic-like sulfur mustard, thereby reducing its ability to alkylate skin proteins. Finally, the formulation of the sponge was modified to include nucleophilic additives to act as a reactive moiety for the sulfur mustard in place of skin proteins. Taken together, the polyurethane sponge was shown to decontaminate and detoxify guinea pig skin exposed to two classes of chemical warfare agents: organophosphates such as GD and alkylating compounds such as HD.

73.6.7 Immobilized enzyme badges (Gordon et al., 2002)

The sponge can incorporate a detection system for organophosphates and alkylating agents—a unique attribute not present in current decontamination methods. The immobilized enzymes provide a detector and a rapid field system capable of identifying the type of organophosphate. In addition to organophosphate detection, a coupled enzyme reaction provides a rapid colorimetric or electrochemical indication of mustard. With the constant threat of chemical warfare or terrorist acts, the development of alternative means of protecting and decontaminating individuals from exposure to CWAs is critical.

Like Diphotérine, which has been evaluated only for sulfur mustard decontamination, the polyurethane enzyme-immobilized sponge is a novel technology that has demonstrated efficacy for organophosphate and sulfur mustard cutaneous poisoning, but is only now

undergoing evaluation for decontamination of biological warfare agents (T-2 mycotoxin, botulinum toxin) and radionucleotides (Gordon et al., 2006). Another advantage of cholinesterases over general reacting additives is that the cholinesterases are the direct target for current or future warfare agents, and therefore countermeasures that incorporate cholinesterases should not require major reformulation. These new technologies likely will provide more efficacious solutions for the soldier in the future.

73.7 Concluding remarks and future directions

Organophosphorus nerve agents are a serious threat to military and civilian personnel. These agents are some of the most potent toxic agents and are specific inhibitors of cholinesterases. Organophosphate nerve agents can exist as a vapor and be inhaled, as a liquid they can contaminate skin, or they can be ingested if food or water is contaminated. Vesicating agents such as sulfur mustard cause irreversible cell damage as a result of rapid alkylation, and were an agent of terror during World War I and more recently in Iran and Iraq. Another serious problem that may be encountered while caring for personnel contaminated with chemical warfare agents is the possibility of cross-contamination of medical providers. In addition, during combat or terrorist acts, individuals might be exposed to chemical toxins before they don their protective gear. Decontamination postexposure has the potential to be an important, integral, and therefore necessary step for medical countermeasures against chemical warfare agents. The products described here must meet several criteria to be effective personal decontaminants and detoxifiers of chemical warfare agents for the soldier, although there is room for improvements; thus novel technologies have been discussed. However, any product must be lightweight for individual use, yet be shelf-stable under environmental conditions found in the field. Decontamination and detoxification products must be readily available and work quickly because of the rapidity with which CWAs cause damage: organophosphates penetrate skin in less than 5 min, and mustard produces irreversible cell damage as a result of alkylation equally rapidly. The product should also be environmentally friendly. In the future, one product should incorporate chemical, biological, and radiological decontamination and, when possible, detoxification. With the constant threat of chemical warfare, terrorist acts, or spills of pesticides, the development of alternative means of protecting and decontaminating individuals from exposure is critical.

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