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Cardioactive Substances that Prolong the Open State of Sodium Channels

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Abbreviations and Symbols

ATX_{II}	Toxin II from *Anemonia sulcata*	h	Inactivation variable of Na channels
cAMP	Cyclic adenosine 3′,5′-monophosphate	m	Activation variable of Na channels
D 600	α-Isopropyl-α-[(N-methyl-N-homoveratryl)-γ-aminopropyl]-3,4,5-trimethoxyphenylacetonitrile (gallopamil)	I_K	Transmembrane K current
		I_{Na}	Transmembrane Na current through Na channels
$ScTX_{II}$	Toxin II from *Androctonus australis* Hector	I_{si}	Transmembrane slow inward (Ca, Na) current
STX	Saxitoxin	P_{Na}	Membrane permeability to Na ions
TTX	Tetrodotoxin	$\dot{V}_{max}$	Maximal rate of depolarization during the upstroke of the action potential
F_c	Peak force of contraction		
ΔF_c	Change in peak force of contraction	$[\ ]_o$	Extracellular concentration
		$[\ ]_i$	Intracellular concentration

1 Introduction

It is now recognized that many toxic substances evolved by plants or animals act by interfering with the excitation process of nerve and muscle cells. Specifically, these compounds alter the rapid and transient increase in membrane permeability to Na ions (P_{Na}), that initiates the action potential. According to the general mode by which toxins modify the Na permeability mechanism, or Na channels, two groups can be distinguished. The first group of substances, including tetrodotoxin (TTX) and saxitoxin (STX), blocks the flow of Na ions through Na channels. The second group of toxins maintains an open form of the Na channel, thereby prolonging the time during which Na ions flow into the cell. This group comprises alkaloids (ceveratrum alkaloids, batrachotoxin, aconitine), the diterpenoid grayanotoxins, and polypeptides from scorpions, sea anemones and coral and contains the most potent and most specific substances known to increase the Na permeability of excitable membranes. Toxins

which prolong the open state of Na channels will be referred to as Na channel-*gate* toxins to denote the specific function of the Na channel affected by these agents, i.e. the gating mechanism which controls the activation and inactivation of Na channels.

While the general pharmacology of one subgroup of Na channel-gate toxins, the ceveratrum alkaloids, has been studied for more than a century, the basic mechanism of their action, as outlined above, has been elucidated only during the last 2 decades following the discovery of the ionic permeability changes responsible for membrane excitation (*Hodgkin* and *Huxley* 1952). For methodological reasons we possess more detailed information concerning the effect of Na channel-gate toxins on nerve fibres and nerve cells in culture than on myocardial and other excitable cells. This review, therefore, includes a brief description of effects on the type of preparation which yielded most information on the mechanism of action at the level of Na channels. For in-depth information of the effects of toxins on Na channels, the reader is referred to the reviews by *Ulbricht* (1969, 1974), *Hille* (1970), *Narahashi* (1974), *Khodorov* (1979), *Ritchie* (1979), *Catterall* (1980), and *Lazdunski* et al. (1980), and to the original literature cited in connection with individual toxins.

This review deals with the direct cardiac effects of Na channel-gate toxins observed in isolated cardiac preparations. The main effects are a positive inotropic effect without a significant chronotropic effect and an arrhythmogenic effect. Early references to the direct cardiac effects of ceveratrum alkaloids are given in the reviews by *Krayer* and *Acheson* (1946) and *Trautwein* (1963). *Trautwein* (1963) also reviewed the literature concerning the cardiac effects of aconitine. The *Benforado* review (1967) includes a chapter on the direct cardiac effects of ceveratrum alkaloids and concludes that "the mechanism by which the veratrum alkaloids exert their positive inotropic action on the heart is not known". The last 15 years have brought considerable progress in our understanding of the basic membrane action of ceveratrum alkaloids and other Na channel-gate toxins and of the relationship between ion fluxes through the sarcolemma and myocardial force of contraction. Perhaps the most impressive result is that the large group of chemically diverse Na channel-gate toxins represents a single class of inotropic agents. They possess a characteristic mode of action that differs from that of other cardioactive drugs, e.g., cardioactive steroids, catecholamines, and other agents thought to act by influencing the cellular concentration of cyclic nucleotides.

In the intact animal organism, the direct cardiac effects of Na channel-gate toxins may not be recognized because of cardiovascular or cardiocardiac reflexes following the stimulation of sensory nerves by the toxins. The *Bezold-Jarisch* effect exemplifies this action for many ceveratrum

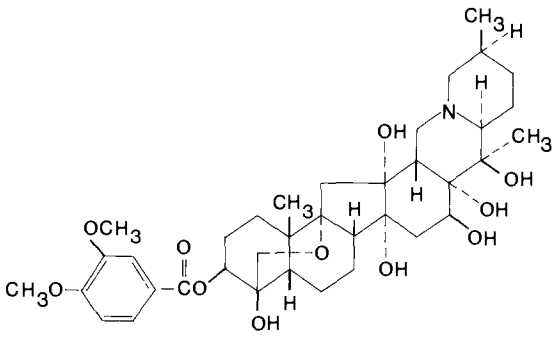
alkaloids (*Krayer* 1961). The hypotensive and other circulatory effects of ceveratrum alkaloids were reviewed by *Benforado* (1967), *Kupchan* and *Flacke* (1967), and *Krayer* and *Meilman* (1977). The older literature on this subject was surveyed by *Krayer* and *Acheson* (1946) in their comprehensive review of the pharmacology of veratrum alkaloids. Notably, certain Na channel-gate toxins lack a hypotensive effect; for example, some natural and semi-synthetic ceveratrum alkaloids (*Kupchan* and *Flacke* 1967) and at least one of the polypeptide toxins, namely anthopleurin A, which has a direct positive inotropic effect on the myocardium in conscious dogs (*Blair* et al. 1978).

2 Chemistry of Na Channel-Gate Toxins

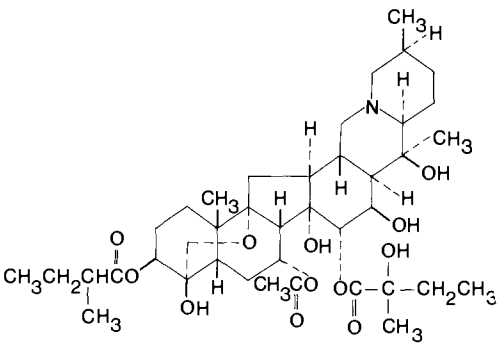
2.1 Alkaloids

2.1.1 Ceveratrum Alkaloids

The veratrum alkaloids are steroid alkaloids that occur in liliaceous plants belonging to the genera *Veratrum, Zygadenus, Stenanthium,* and *Schoenocaulon.* These genera comprise a section of the suborder *Melanthioideae* (*Kupchan* et al. 1961). The terms *ceveratrum* and *jerveratrum* alkaloids were introduced to distinguish two chemically and pharmacologically different groups of veratrum alkaloids (*Fieser* and *Fieser* 1959). The jerveratrum alkamines are secondary amines, contain only one to three atoms of oxygen, and are found in unhydrolyzed plant extracts in part as the free alkamines and in part in combination with one molecule of D-glucose as glucoalkaloids. Jerveratrum alkaloids do not prolong the open state of Na channels. Some jerveratrum alkaloids, such as veratramine, antagonize the effect of ceveratrum alkaloids (*Krayer* and *George* 1951), probably because they block Na channels (*Ohta* et al. 1973). The ceveratrum alkamines are tertiary amines, are highly hydroxylic, and contain seven to nine atoms of oxygen. They usually occur esterified with various acids as ester alkaloids, but are sometimes unconjugated. They have never been found as glycosides. The ceveratrum alkaloids can be formally derived from (22 S, 25 S)-5β-cevanine. Their chemistry has been reviewed by *Kupchan* and *By* (1968). The structures of the monoester veratridine and the triester germitrine are shown in Fig. 1. The name *veratrine* is usually used for the total alkaloids of the seeds of *Schoenocaulon officinale;* it does not refer to a well-defined preparation of constant composition (*Krayer* and *Acheson* 1946; *Kupchan* et al. 1961). The use of this and other mixtures of veratrum alkaloids for scientific purposes is discouraged. Jerveratrum alkaloids may antagonize the effect of ceveratrum



Veratridine



Germitrine

Fig. 1. Chemical structures of the ceveratrum alkaloids veratridine and germitrine. (Drawn after *Kupchan* and *By* 1968)

alkaloids, and there are more subtle differences between the effects of individual ceveratrum alkaloids (veratridine versus cevadine; *Reiter* 1963; veracevine and germine and their esters, Sect. 3.2; veratridine versus germitrine, Sect. 4.2.5).

2.1.2 Batrachotoxin

Batrachotoxin is the most toxic of the steroid alkaloids extracted from the skin of the Columbian arrow poison frog *(Phyllobates aurotaenia).* It is a pyrrolecarboxylate ester of the pregnane derivative batrachotoxinin A (*Tokuyama* et al. 1969).

2.1.3 Aconitine

Aconitine is the main alkaloid of *Aconitum napellus* of the plant family *Ranunculaceae* (*List* and *Hörhammer* 1969). Like the ceveratrum alkaloids, aconitine is an ester of an alkamine with organic acids. The alkamine,

aconine, contains two cyclopentane rings, one piperidine ring, and three cyclohexane rings. In aconitine, aconine is esterified with benzoic acid and acetic acid. The structure of aconitine was reported by *Wiesner* et al. (1969).

2.2 Diterpenoids

Gyanotoxins are the toxic principles found in the leaves of various species of *Rhododendron, Kalmia,* and *Leucothoe* (Ericaceae). They are diterpenoids with a perhydroazulene skeleton and their chemical structures have been determined (*Kakisawa* et al. 1965; *Kumazawa* and *Iriye* 1970).

2.3 Polypeptides

2.3.1 Scorpion Toxins

Toxins of North African scorpions were first purified, characterized, and sequenced by *Rochat, Miranda, Lissitzky,* and colleagues (*Miranda* et al. 1970; *Rochat* et al. 1970; *Kopeyan* et al. 1979). These toxins are basic polypeptides with molecular weights of approximately 7000. Each scorpion species studied contained multiple toxins having extensive sequence homology (*Rochat* et al. 1970). The molecular weight of toxin II from *Androctonus australis* HECTOR ($ScTX_{II}$) is 7249 (*Miranda* et al. 1970).

2.3.2 Sea Anemone Toxins

Anemone toxins were first isolated in pure form from *Anemonia sulcata,* shown to be basic polypeptides of 2500–5000 molecular weight and sequenced by *Béress, Wunderer,* and their colleagues (*Béress* et al. 1975a,b, 1977; *Wunderer* et al. 1976). The sequences of three toxins from *Anemonia sulcata* and one from *Anthopleura xanthogrammica* were determined (*Wunderer* et al. 1976; *Béress* et al. 1977; *Martinez* et al. 1977; *Wunderer* and *Eulitz* 1978; *Tanaka* et al. 1977). Toxins I and II from *Anemonia sulcata* and anthopleurin A from *Anthopleura xanthogrammica* consist of 47–49 amino acid residues and have substantial sequence homology. None of the anemone toxins has detectable sequence homology when compared to the scorpion toxins.

2.3.3 Coral Toxin

Goniopora toxin, a polypeptide with a molecular weight of 12 000, was isolated and purified from the coral *Goniopora* spp. (*Hashimoto* and *Ashida* 1973).

3 Mode of Action of Na Channel-Gate Toxins

3.1 Methods of Analysis

Different methods have been employed to study the effects of drugs on Na channels; electrophysiologic analysis of membrane voltage and membrane current, isotopic flux measurements of ion permeability, and direct binding studies of radiolabelled toxins. The results obtained with the latter two methods were recently reviewed (*Catterall* 1980), and have helped significantly to elucidate molecular aspects of the effects of toxins on the Na channel. The electrophysiologic approach, with its advantage of high time resolution, provides information allowing us to predict how the Na channel-gate toxins modify the transmembrane electrical activity of cells. This is particularly important for an understanding of the cardiac effect of these agents. The effects on the kinetics of Na channels are therefore reviewed in some detail. It has become customary to analyse the effects of drugs on Na channels in terms of the conceptualization introduced by *Hodgkin* and *Huxley* (1952) to describe the normal Na permeability changes in the squid giant axon. According to this theory, two voltage- and time-variant parameters, the activation variable m and the inactivation variable h, govern the changes of the Na permeability during excitation. The Na permeability is turned on because of an increase of m if the membrane is depolarized, and the turning off during maintained depolarization is due to a decrease of h. Repolarization turns off P_{Na} because of a decrease in m. This analysis was also used with some modifications to describe the behaviour of Na channels in other types of excitable cells, including cardiac cells (see *Trautwein* 1973). The hypothetical subunit of the Na channel, which gives adequate kinetics of opening and closing, is referred to as the gating mechanism, consisting of the m gate and the h gate.

3.2 Ceveratrum Alkaloids

The veracevine ester veratridine has received the most attention among the various ceveratrum alkaloids. A detailed analysis of the effect of veratridine on the frog node of Ranvier was performed by *Ulbricht* (1969, 1972a,b) using the voltage-clamp technique. Normally, the Na permeability of nerve fibres exhibits a fast and transient rise of about 1 ms duration during a voltage-clamp depolarization. In contrast, the P_{Na} observed in the veratridine-treated node, in addition to the normal Na permeability, develops over a period of several seconds during depolarization and is maintained as long as the membrane is clamped at the depolarized level.

The veratridine-induced P_{Na} was quantitatively described by introducing an activation variable s (analogous to m) exhibiting the potential- and time-dependence observed and by omitting an inactivation term corresponding to h (*Ulbricht* 1969). For a stepwise depolarization, the time course of s follows the equation $s_{(t)} = s_{(t=\infty)} (1 - e^{-t/\tau_s})$, where t is the time after the start of the depolarization and τ_s is a time constant. The time constant of activation of the veratridine-induced P_{Na} (τ_s) is of the order of seconds, i.e. about 10^4-times larger than τ_m, the time constant of the normal P_{Na} activation. The voltage-dependence of the veratridine-induced P_{Na} activation is less steep and shifted to more negative membrane potentials compared to that of normal P_{Na}.

Veratridine seems to modify some of those Na channels participating normally in the production of the action potential rather than to create new channels (*Ulbricht* 1969). This would explain why the appearance of the alkaloid-induced P_{Na} is accompanied by a decrease in the normal transient P_{Na}. Furthermore, normal and modified channels distinguish to comparable degrees between Na, Li, choline, and tromethamine, and both are blocked by TTX at rates and to extents that are very similar (*Ulbricht* 1974). The concentration of veratridine appears to determine the proportion of Na channels that is modified, while at the level of the single Na channel the interaction with veratridine appears to be a reversible all-or-none event (*Ulbricht* 1972a).

At concentrations exceeding about 10 µmol/litre veratridine induces a sustained depolarization of the node of Ranvier due to an increase in P_{Na} (*Ulbricht* 1969). It is caused by the two effects of veratridine on Na channels; block of inactivation and shift of activation to more negative potentials, thus allowing the spontaneous opening of Na channels at the level of the normal resting potential. Some pharmacologically important effects of veratridine occur at lower concentrations when the resting membrane potential shows very little, if any depolarization. At this stage of veratridine action, the presence of modified Na channels is apparent as a distinct delay in the final phase of repolarization of the action potential (after-depolarization). The effect reflects the large value of τ_s that determines the rate at which modified Na channels deactivate after repolarization (*Ulbricht* 1969). By this mechanism, veratridine greatly prolongs the phase of increased P_{Na} and Na influx associated with each action potential.

Other ceveratrum alkaloids are similar to veratridine in producing after-depolarizations in nerve fibres, but individual alkaloids differ with respect to duration (*Graham* and *Gasser* 1931; *Shanes* 1952; *Honerjäger* 1973). The alkamines germine and veracevine induce after-depolarizations of relatively short duration in crustacean nerve axons. The half-life of after-depolarization decay is 6–8 ms (*Honerjäger* 1973). In the same

study it was shown that number and nature of the ester groups attached to the alkamine modify the duration of after-depolarization. For example, replacement of the 3β-hydroxyl in veracevine by an acetyl (cevacine), angeloyl (cevadine), or veratroyl group (veratridine) causes a progressive prolongation of the after-depolarization up to about 3 s (half-life) in the case of veratridine. Even longer-lasting after-depolarizations were observed with the di-, tri-, and tetraester alkaloids in guinea-pig papillary muscle (Sect. 4.2.5). The semisynthetic germine ester germine-3-acetate produces an after-depolarization in curstacean nerve fibres that decays with a half-life of 40–70 ms. Voltage-clamp experiments on squid axons showed that this alkaloid induces a TTX-sensitive slow component of Na permeability (*Honerjäger* 1973). After repolarization this component of P_{Na} deactivates about 20-times faster than the P_{Na} induced by veratridine. This may indicate that all the various ceveratrum alkaloids react with Na channels, but differ in the degree to which they slow the *m*-gating process.

3.3 Batrachotoxin

A detailed voltage-clamp analysis carried out on the frog node of Ranvier showed that batrachotoxin alters both the gating mechanism and ion selectivity of Na channels (*Khodorov* and *Revenko* 1979). The Na channels lose the ability to inactivate, and the voltage-dependence of activation is shifted by about 50 mV to more negative voltages. Therefore, a fraction of Na channels is permanently activated at the normal level of resting potential, resulting in depolarization. The time constant of activation is also affected by batrachotoxin, but to a much lesser extent than by veratridine. Batrachotoxin increases the relative permeability of the Na channels toward larger ions, including Ca ions. This finding, by *Khodorov* and *Revenko* (1979), is of particular interest with regard to the positive inotropic effect on myocardial cells of batrachotoxin, as well as other Na channel-gate toxins (Chap. 4). If myocardial Na channels were to lose their ionic selectivity and become significantly permeable to Ca ions, this would explain a positive inotropic effect of the Na channel-gate toxins. It seems, however, that this effect of batrachotoxin on neural Na channels cannot be extended to cardiac Na channels, at least as far as the action of veratridine and $ScTX_{II}$ is concerned. The Ca influx induced by these Na channel-gate toxins in cardiac cells in culture does not occur through Na channels, but by way of Na-Ca exchange (Sect. 5.3.2).

Khodorov and *Revenko* (1979) further showed that the modification of Na channels by batrachotoxin requires a specific functional state of the Na channels, namely their open configuration. Thus the effect develops

much more rapidly if the node of Ranvier is stimulated repetitively in such a way that the Na channels are not inactivated by the holding potential and the pulses are large enough to activate Na channels. The concentration-effect relationship suggests that each channel receptor binds only one molecule of batrachotoxin. A study of the effect of batrachotoxin on the action potential of guinea-pig papillary muscle led to the same conclusion (Sect. 4.3.2).

3.4 Aconitine

The mode of action of aconitine on Na channels was elucidated in voltage-clamp experiments on the frog node of Ranvier (*Schmidt* and *Schmitt* 1974; *Mozhayeva* et al. 1977), and was found to be very similar to that of batrachotoxin. Aconitine-modified Na channels lose the property of inactivation and open at more negative potentials than the normal resting potential. These channels open with a time constant about twice as large as normal Na channels. As with batrachotoxin, the development of the effect is speeded up by periodical depolarizations if they lead to an activation of Na channels. This suggests that the open configuration of the Na channel gate is more favourable for the interaction with aconitine. Aconitine, like batrachotoxin, reduces the selectivity of Na channels for monovalent cations (*Mozhayeva* et al. 1977).

3.5 Grayanotoxins

Grayanotoxins have been found to depolarize the membranes of various excitable cells (see *Starkus* and *Narahashi* 1978). As shown on the squid giant axon, this is due to a selective and TTX-sensitive increase in resting P_{Na} (*Narahashi* and *Seyama* 1974). The effect of grayanotoxins on the time- and voltage-dependence of activation and inactivation of Na channels has not been published in detail.

3.6 Scorpion Toxins

Venom of *Leiurus quinquestriatus* delays the repolarization of the action potential, reduces the resting membrane potential and induces spontaneous activity in myelinated nerve fibres (*Adam* et al. 1966). Voltage-clamp experiments on the frog node of Ranvier showed that the main effect of the venom is to slow the inactivation of Na channels (*Koppenhöfer* and *Schmidt* 1968a,b). Venoms or toxins of other North African scorpions

(*Buthus thamulus, B. eupeus, Androctonus australis* HECTOR) have the same principal effect on nerve fibres (see *Khodorov* 1979). In the squid giant axon, which allows application of substances to either side of the axolemma, venom of *B. thamulus* is effective only if applied from the extracellular side (*Narahashi* et al. 1972). More recently, *Catterall* (1980) showed that a toxin purified from *L. quinquestriatus* venom increases the fraction of Na channels activated by veratridine, aconitine, or grayanotoxin I in cultured neuroblastoma cells: His findings indicate that scorpion toxin and the group of alkaloidal and diterpenoid toxins (lipid-soluble toxins) bind to separate receptor sites of the Na channel which interact allosterically.

In contrast to the venoms of North African scorpions, which modify Na inactivation, venom of the American scorpion *Centruroides sculpturatus* alters the activation gate without modifying the *h* gate in the frog node of Ranvier (*Cahalan* 1975). This venom causes a shift of the activation voltage-dependence by 40–50 mV in the hyperpolarizing direction, while the modification of the Na channel requires depolarization.

While many Na channel-gate toxins alter the function of isolated cardiac muscle by modifying sarcolemmal Na channels, scorpion toxins seem to act mainly by releasing neurotransmitters from intracardiac nerve fibres, i.e. by their effect on neural Na channels (Sect. 4.6).

3.7 Sea Anemone Toxins

3.7.1 Toxin II from Anemonia sulcata (ATX_{II})

In the crayfish giant axon, ATX_{II} has little or no effect on resting potential, but produces a marked prolongation of the falling phase of action potentials (*Rathmayer* and *Béress* 1976). As shown in the frog node of Ranvier, and crayfish giant axons, the main effect of ATX_{II} is to slow the rate of the Na channel inactivation (*Bergman* et al. 1976; *Romey* et al. 1976). The activation of Na channels is not markedly altered. The curve relating steady-state inactivation to membrane potential shows a decrease in slope under the influence of ATX_{II}, so that moderate depolarizations inactivate a larger than normal fraction of Na channels. The receptor for ATX_{II} does not seem to be accessible from inside the axolemma. Like scorpion toxin, ATX_{II} increases the fraction of Na channels modified by veratridine (*Catterall* 1980). Scorpion toxin and ATX_{II} appear to act at a common receptor site associated with the Na channel (*Catterall* 1980).

3.7.2 Anthopleurin A

Low concentrations of anthopleurin A produce slow transient depolarizations and a prolongation of the repolarization phase in the crayfish giant axon, while high concentrations induce a sustained depolarization (*Low* et al. 1979). Voltage-clamp experiments show that anthopleurin A alters the kinetics of Na channels (*Low* et al. 1979). Modified Na channels are activated at more negative membrane potentials, and the inactivation is slowed as is the removal of activation after repolarization.

3.8 Coral Toxin

Goniopora toxin prolongs the falling phase of the action potential in rabbit myocardium (*Fujiwara* et al. 1979), and this effect is abolished by TTX. This is so far the only available evidence indicating that the toxin slows the kinetics of Na channels.

3.9 Silent Na Channels

The basic effect of the various Na channel-gate toxins was discovered in excitable membranes that produce their action potential by the activation of Na channels. However, it is becoming increasingly evident that neither the presence of a Na channel-dependent mechanism of excitation, nor excitability as such are prerequisites for Na channel-gate toxins to cause the appearance of persistently activated Na channels. Veratridine opens Na channels in the pancreatic β-cell membrane which normally produces impulses by the activation of Ca channels (*Donatsch* et al. 1977). The action potential of chick embryonic heart cells in an early stage of development is due to the activation of TTX-insensitive, slow Na-Ca channels. Nevertheless, veratridine depolarizes these cells and the effect is blocked by TTX (*Sperelakis* and *Pappano* 1969; *Bernard* and *Couraud* 1979; *Romey* et al. 1980). Polypeptide toxins also produce bioelectric effects that are blocked by TTX in slow cells of the chick embryo heart (*Bernard* and *Couraud* 1979; *Romey* et al. 1980). These effects are described in detail in Chap. 4. Veratridine produces a TTX-sensitive depolarization in non-spiking sensory dendrites of the crab (*Lowe* et al. 1978). In an inexcitable cell line derived from a rat brain tumor, veratridine or polypeptide toxins open TTX-sensitive Na channels (*Romey* et al. 1979). When exposed to Na channel-gate toxins, fibroblasts exhibit an increased uptake of Na that is blocked by TTX (*Munson* et al. 1979; *Pouysségur* et al. 1980). There are conflicting reports as to whether Na channel-gate toxins open Na channels in glial cells (*Villegas* et al. 1976; *Tang* et al. 1979).

As it is highly unlikely that the Na channel-gate toxins create Na-selective and TTX-sensitive ionic channels in these membranes, it is generally assumed that Na channels pre-exist in a state which under normal conditions precludes their electrical activation. The term silent Na channels has been suggested by *Romey* et al. (1979). If these Na channels were in a state characterized by faster inactivation kinetics than activation kinetics, this would explain why they are normally electrically silent, but able to open in the presence of a toxin that specifically slows the inactivation (*Romey* et al. 1979).

3.10 Antagonists

3.10.1 Non-Competitive Antagonists

TTX and STX are potent and selective blockers of Na channels and they have no other known action (*Narahashi* 1974). TTX and STX also block the flow of Na ions through Na channels modified by Na channel-gate toxins, and thus are specific antagonists of Na channel-gate toxins. The blockers and the gate toxins modify different properties of the Na channel. TTX and STX, in partially blocking concentrations, reduce the total available Na permeability, but do not alter the kinetics of the unblocked Na channels. In contrast, the gate toxins alter the kinetic (m and/or h) behaviour of the Na channels. This difference suggests different receptor sites at the Na channel for blockers and gate toxins respectively, and thus a non-competitive type of antagonism. *Catterall* (1975) investigated the nature of this antagonism by using the initial rate of Na influx into cultured neuroblastoma cells to quantify the effect of veratridine or batrachotoxin. He has shown that activation of Na influx by the Na channel-gate toxins is non-competitively inhibited by TTX. Further proof that the receptors for TTX and Na channel-gate toxins are not identical was obtained in experiments on the specific binding of labelled TTX to nerve membrane preparations. This binding (and that of labelled STX) was unaffected even by high concentrations of Na channel-gate toxins (see *Catterall* 1980).

Although the TTX-sensitivity of biological effects points to the involvement of Na channels, there are some instances in which the lack of sensitivity to TTX does not rule out the participation of Na channels. Examples of cells possessing TTX-insensitive Na channels are denervated skeletal muscle fibres (*Redfern* et al. 1970) and giant neurons of the snail *Helix aspersa* (*Chamberlain* and *Kerkut* 1969). These TTX-insensitive Na channels are susceptible to the action of Na channel-gate toxins such as veratridine (*Leicht* et al. 1971) or batrachotoxin (*Albuquerque* and *Warnick* 1972).

Normal Na channels and those modified by veratrine are blocked by the local anaesthetic lidocaine (frog node of Ranvier; *Hille* 1968). Lidocaine and tetracaine inhibit veratridine-induced Na uptake by embryonic cardiac cells (*Fosset* et al. 1977).

An increase in external Ca concentration is known to reduce the depolarizing effect of veratridine (*Ulbricht* 1969). To understand this antagonism it is necessary to recall the effect of external Ca ions on normal Na channels. An increase in $[Ca]_o$ shifts the voltage dependence of both activation and inactivation to less negative membrane potentials (*Frankenhaeuser* and *Hodgkin* 1957). Varatridine-modified Na channels lose the inactivation gate, but their activation gate is similarly sensitive to changes of $[Ca]_o$ as that of normal Na channels (frog node of Ranvier; *Ulbricht* 1969). Thus, increasing $[Ca]_o$ at a given level of depolarization reduces the fraction of activated veratridine-modified Na channels and may lead to repolarization. This may also explain why concentrations of external Ca higher than 2 mmol/litre inhibit the veratridine-induced Na uptake by embryonic cardiac cells (*Fosset* et al. 1977). It is not necessary to assume a specific, e.g. competitive, interaction between Ca ions and veratridine to account for this antagonism. However, the inhibition by external Ca ions of the veratridine-induced Na influx into neuroblastoma cells (*Catterall* 1975) and cardiac cells in culture (*Galper* and *Catterall* 1979) appears to be of the competitive type.

3.10.2 Competitive Antagonists

The clarification of a pharmacological antagonism usually requires establishing complete concentration-effect relationships in systems where the effect is a more or less direct measure of the degree of receptor occupancy by the drug. In the case of Na channel-gate toxins, *Catterall* (1975) was the first to obtain complete concentration-effect relationships for their effect on Na permeability by using the stimulation of ^{22}Na influx in cultured neuroblastoma cells. With this system he showed that there are competitive interactions among veratridine, batrachotoxin, aconitine, and grayanotoxin I (see *Catterall* 1980). The effect of saturating concentrations of batrachotoxin is *reduced* by each of the other lipid-soluble toxins, suggesting that batrachotoxin is a full agonist, whereas veratridine, aconitine, and grayanotoxin are partial agonists acting at a common receptor site of the Na channel. A competitive antagonist without noticeable intrinsic effect on the Na channels has apparently not yet been found.

4 Cardiac Action of Na Channel-Gate Toxins

4.1 Role of Na Channels in the Cardiac Action Potential

4.1.1 Na Channels in Different Cell Types of the Heart

During the last 15 years the application of voltage-clamp techniques to cardiac tisssues has provided much information on the transmembrane ionic currents underlying the action potential of cardiac cells, which is important for an understanding of the cardiac action of Na channel-gate toxins. The reader is referred to the reviews by *Trautwein* (1973) and *Reuter* (1979) and to the articles by *McAllister* et al. (1975) and by *Beeler* and *Reuter* (1977). In the case of mammalian *ventricular* myocardium, the action potential has been shown to result from the sequential and transient activation of a fast inward Na current (I_{Na}), a secondary, slow inward current carried by Ca and Na ions, (I_{si}), and an outward K current (I_K) (*Trautwein* 1973; *Reuter* and *Scholz* 1977; *McDonald* and *Trautwein* 1978). The fast P_{Na} system responsible for I_{Na} is very similar to that of nerve cells and the term Na channels, originally applied to the ionic pathwahs of the Na conductance mechanism of nerve cells (*Hille* 1970), is now also used for that of cardiac cells (*Hondeghem* and *Katzung* 1977). The rapid upstroke of the action potential is caused by the rapid increase of I_{Na}, and the first time derivative of membrane potential during the upstroke ($\dot{V}$) provides an indirect estimate of I_{Na} (*Reuter* 1979; *Walton* and *Fozzard* 1979). The electrotonic current resulting from the potential change during the upstroke is the stimulus for excitation of adjacent cells. Therefore, I_{Na} is the basis for conduction of the action potential, and conduction velocity is largely determined by the $\dot{V}_{max}$ of the action potential. I_{Na} subsides completely within a few milliseconds after its activation, but the membrane potential remains at a positive level because of the now-apparent activation of I_{si}. The subsequent repolarization process is determined by the inactivation of I_{si} and the activation of I_K. As in ventricular myocardial cells, a fast, inward Na current is responsible for the upstroke of the action potential in *Purkinje* fibres and *atrial* muscle fibres (*Trautwein* 1973). The action potential of Purkinje fibres exhibits a higher value of $\dot{V}_{max}$ than the fibres of the working myocardium. This allows the Purkinje fibre to play its physiological role in distributing excitation, leading to the nearly simultaneous contraction of the different parts of the ventricles. In Purkinje fibres, I_{Na} is *not* completely inactivated after the upstroke and participates in the ionic currents flowing during the plateau phase (*Attwell* et al. 1979). Thus, the action potential duration of Purkinje fibres, but not of ventricular muscle fibres, is markedly shortened by the specific Na channel blocker TTX

(*Coraboeuf* et al. 1979). The slowly rising action potentials of cells of the *sinoatrial node* are insensitive to TTX (*Yamagishi* and *Sano* 1966) indicating that Na channels are not involved in producing the rising phase of the action potential. Yet the presence of Na channels in this cell type is indicated by the observation that hyperpolarization of pacemaker cells by application of carbachol produces an increase in $\dot{V}_{max}$ of the action potential that is entirely suppressed by TTX (*Kreitner* 1975). Hyperpolarization by the voltage-clamp technique also reveals a fast Na current that is inactivated by depolarization and blocked by TTX (*Noma* et al. 1977). The upstroke of the action potential of *atrioventricular nodal* fibres shows two components (*Ruiz-Ceretti* and *Ponce Zumino* 1976). The first of these is attributed to the activation of Na channels because it depends strongly on $[Na]_o$ and is inhibited by TTX. Its relatively low $\dot{V}_{max}$ of about 13 V/s is accounted for by the low resting potential of nodal cells causing inactivation of the Na channels. The voltage-dependence of $\dot{V}_{max}$ of atrioventricular nodal action potentials corresponds to that of Na channels (*Shigeto* and *Irisawa* 1974). The second component of the action-potential upstroke is attributed to the slow inward current (*Ruiz-Ceretti* and *Ponce Zumino* 1976).

In summary, electrophysiologic experiments revealed the presence of Na channels in the plasmalemma of cells of the sinoatrial and atrioventricular nodes, atrial and ventricular myocardium, and of Purkinje fibres. Na channel gate toxins are expected to modify the electrical activity of all cells possessing Na channels unless the Na channels lack the pharmacologic receptor for alteration of the gating mechanism by the toxin.

The effects of Na channel-gate toxins on membrane currents of cardiac cells under voltage-clamp conditions are largely unknown. However, there is substantial indirect evidence obtained by intracellular potential recording suggesting that these toxins affect the kinetics of I_{Na} in a way similar to their effect on neural Na channels. A slowing of I_{Na} inactivation, as produced in the nerve membrane by sea anemone or scorpion toxins, or the removal of inactivation combined with a slowing of activation in a fraction of Na channels, as produced by veratridine in the node of Ranvier, would add a component of Na current to the membrane currents flowing during the plateau and repolarization phases of the cardiac action potential. This should drive the membrane potential from the normal level determined by the balance of I_{si} and I_K towards the equilibrium potential for Na ions (approx. + 50 mV) until the delayed inactivation of the modified Na channels and normal repolarizing mechanisms allow a return of the membrane potential to the normal resting level. Indeed, a prolongation of the repolarization phase is the characteristic effect on cardiac cells of many Na channel-gate toxins. Strong evidence for a selective modification of I_{Na} by these agents is the prevention of

their effect on the action potential in Na-poor medium or in the presence of TTX, i.e. under conditions where I_{Na} is absent and the action potential occurs through the activation of I_{si}.

4.1.2 Delayed Repolarization and Oscillatory After-Potentials

The repolarization of the cardiac action potential proceeds in certain phases that are affected differently by individual Na channel-gate toxins. The first or early phase of repolarization to be distinguished in Purkinje fibres occurs immediately after the rapid upstroke and extends from the spike to the beginning of the plateau phase. This early repolarization, which is absent or much less pronounced in atrial and ventricular muscle fibres, has received scant attention in the study of Na channel-gate toxins and seems to be little affected by these agents (*Schmidt* 1960; *Heistracher* and *Pillat* 1962; *Hogan* and *Albuquerque* 1971; *Arbel* et al. 1975; *Shimizu* et al. 1979). The early phase of repolarization is followed by the plateau phase and a phase of rapid repolarization during which the resting potential level is approached. In purely descriptive terms, there are two ways in which the course of repolarization following the plateau phase may be prolonged, and Na channel-gate toxins can be classified on this basis. First, repolarization can be slowed by a prolongation of the plateau phase, i.e., by a broadening of the action potential at nearly all potential levels between the peak of the plateau potential and the resting potential. Second, repolarization can be slowed, more or less abruptly, at any point during the fast phase of repolarization which follows the plateau phase; the latter and a variable, initial part of the former then remain unaffected. This second modification is usually called depolarizing after-potential or after-depolarization. These terms should replace the term negative after-potential (e.g., Fig. 1 in *Shanes* 1958), which was introduced when electrical activity was measured with extracellular electrodes. The intracellular potential is changed in the positive direction during an after-depolarization.

It should be noted that a slowing of the rate of repolarization may have either an *anti-arrhythmic* or an *arrhythmogenic* effect, depending upon the level of membrane potential at which the slowing occurs. A broadening of the plateau phase at near-zero membrane potential, where both I_{Na} and I_{si} are inactivated in the course of the action potential and unable to recover from inactivation, will make the cell refractory against premature action potentials for as long as the membrane is kept at this depolarized level. If, however, the rate of repolarization is slowed at more negative levels of membrane potential (negative to about -20 mV), spontaneuous action potentials often arise from the prolonged repolarization phase (*Cranefield* 1977). This is probably the most important cause

of the arrhythmias produced by Na channel-gate toxins. The coupling interval and the shape of the triggered action potential depend on the recovery kinetics of I_{Na} and I_{si} during the after-depolarization. As shown by *Gettes* and *Reuter* (1974) this recovery is voltage dependent, the time constant for recovery of $\dot{V}_{max}$ increasing from less than 20 ms at −80 mV to more than 100 ms at −60 mV. When the membrane is further depolarized to −9 mV, I_{Na} is completely inactivated and the time constant for recovery of I_{si} increases from about 60 ms to about 250 ms. The critical toxin-induced prolongation of the repolarization phase which leads to re-excitation will therefore depend on the level of membrane potential at which the individual agent slows repolarization. In the case of aconitine (see following sections), which slows repolarization at about −60 mV, triggered activity occurs as soon as the action potential is prolonged by only about 30 ms (cat papillary muscle; *Heistracher* and *Pillat* 1962). In contrast, veratridine (see following sections), which prolongs the repolarization phase already at −20 mV, induces spontaneous activity after prolonging the action potential by more than 400 ms (guinea-pig papillary muscle; *Honerjäger* and *Reiter* 1975).

After-depolarizations, which result from a delay of the repolarization process, have to be distinguished from *oscillatory after-potentials,* which occur after the membrane has repolarized completely (*Bozler* 1943; *Ferrier* 1977). The latter phenomenon is frequently observed in cardiac cells under conditions of Ca overload and in association with after-contractions (*Reiter* 1962; *Kaufmann* et al. 1963). Oscillatory after-potentials result from an oscillatory inward current that is thought to be triggered by a phasic release of Ca ions from an intracellular store (*Kass* et al. 1978a,b). Thus, an oscillatory Ca release from an intracellular store is regarded as the cause for both after-contractions and oscillatory after-potentials. *Kass* et al. (1978b) suggested that the oscillatory inward current may represent either the flow of cations through a non-selective ion channel or the inward movement of Na ions via an alectrogenic Na-Ca exchange mechanism activated by the rise in free intracellular Ca concentration. Although oscillatory after-potentials are inhibited by the specific Na channel blocker TTX (*Kass* et al. 1978b; *Rosen* and *Danilo* 1980), oscillatory after-potentials are not likely to be mediated by a delayed reopening of Na channels. The work by *Kass* et al. (1978b) suggests that any intervention which suppresses the intracellular Ca release responsible for the oscillatory after-potentials will also inhibit oscillatory after-potentials. TTX has a negative inotropic action (Sect. 4.2.5) and may thus inhibit oscillatory after-potentials indirectly by reducing intracellular Ca release.

Cranefield (1977) has extended the term after-depolarization to include oscillatory after-potentials. He distinguishes between "early after-depolari-

zations", which interrupt the repolarization phase of the action potential, and "delayed after-depolarizations", which follow complete repolarization.

4.2 Ceveratrum Alkaloids

4.2.1 Sinoatrial Node

There are no reports on the effects of ceveratrum alkaloids on intracellular potentials in the sinoatrial node, but there is some information on the effects on atrial rate in the dog heart-lung preparation and in isolated guinea-pig atria. *Benforado* (1957) investigated the effects of nine ceveratrium ester alkaloids (veratridine, cevadine, veratroylzygadenine, vanilloylzygadenine, germbudine, neogermitrine, germitetrine, protoveratrine A, and protoveratrine B) on the dog heart-lung preparation by means of electrocardiographic (ECG) recording. Only cevadine produced a consistent chronotropic effect, i.e. a sinus tachycardia. The general lack of chronotropic activity shows clearly that the marked bradycardia seen following administration of ester alkaloids to the intact animal has no direct cardiac component (*Benforado* 1957). The mechanism of the positive chronotropic effect of cevadine is unknown. Cevine, the 3-α-hydroxyl isomer of the alkamine veracevine, causes a bradycardia in the dog heart-lung preparation (*Moe* and *Krayer* 1943) by an unknown mechanism. Germine-3-acetate (31–540 μmol/litre) has a positive chronotropic effect on isolated guinea-pig atrium that is abolished by 0.5 μmol/litre propranolol (*Seifen* 1969). This may indicate that germine-3-acetate affects the sinoatrial node by releasing catecholamines from intracardiac nerve fibres.

4.2.2 Atrium

Horackova and *Vassort* (1973, 1974) analysed the effect of the alkaloid mixture veratrine (veratrine sulphate, Sigma) on small atrial bundles of the frog heart. They used the double sucrose-gap technique to record both membrane potential and membrane current under membrane-potential control. Veratrine prolongs the repolarization phase of the action potential, which agrees with the earlier findings that veratrine and one of its constituents, cevadine, prolong the action potential in isolated dog and rat atrial tissue (*Brooks* et al. 1955; *Matsuda* et al. 1953). The addition of TTX (3 μmol/litre) completely suppresses the effect of veratrine on the action potential of frog atrial preparations. Under voltage-clamp conditions, veratrine prolongs the phase of inward current associated with moderate step depolarizations from the normal resting potential, and this induced inward current decays only slowly after repolarization

(inward "tail" current). The veratrine-induced inward current is abolished by TTX (3 μmol/litre), and veratrine fails to modify the remaining inward current (I_{si}) if external Na ions are replaced by sucrose. If Na ions are replaced by Li, veratrine increases inward current, as in the presence of external NA. These findings indicate that veratrine prolongs the open state of Na channels in frog atrial fibres. During a depolarizing pulse, the decay of I_{Na} is slowed by veratrine, indicating that the *h* gate closes more slowly in a fraction of Na channels. In the modified channels, the time constant of inactivation (τ_h) during depolarization by 40 mV from the normal resting potential is increased to 1.1–1.9 s (18°–20°C), i.e. by 3 orders of magnitude. There is no obvious effect on the activation of Na channels upon depolarization, but the removal of activation after repolarization is slowed, as indicated by the inward tail currents that decline exponentially with time constants of 0.4–0.7 s. This shows that veratrine has little or no effect on the rate constant α_m, but markedly reduces β_m [1]. These effects are not identical to the effect of veratridine on Na channels in the frog node of Ranvier (Sect. 3.2), although in both types of cells, veratridine and veratrine, respectively, increase the Na inward current associated with the action potential. In the frog node of Ranvier, veratridine diminishes both α_m and β_m, i.e, τ_m is increased during depolarization as well as after repolarization [2]. Furthermore, veratridine completely removes inactivation in the neural Na channels. It is not clear whether this discrepancy reflects a genuine difference between neural and myocardial Na channels or whether the cardiac effects are due to ceveratrum alkaloids other than veratridine present in the alkaloidal mixture veratrine.

Horackova and *Vassort* (1973, 1974) were the first to show that the effect on sarcolemmal P_{Na} and the positive inotropic effect of veratrine are intimately linked. They found that the positive inotropic effect is abolished by TTX (3 μmol/litre), which also blocks the veratrine-induced prolongation of the action potential. The positive inotropic effect of veratrine on frog atrial muscle develops during identical repetitive depolarizations under voltage-clamp conditions indicating that it is related to the change of membrane *current* and not to the veratrine-induced alteration of the duration of the action *potential*. As shown by *Horackova* and *Vassort* (1974) the veratrine-induced Na inward current does not lead to an increase in the rate of force development of the *simultaneous* contraction within a given excitation-contraction cycle. In a previously resting

1 α_m and β_m are the *rate* constants for the activation and deactivation, respectively, of the *m* gate of the Na channels (*Hodgkin* and *Huxley* 1952). These rate constants are related to the *time* constant of the *m* gate by the equation $\tau_m = 1/(\alpha_m + \beta_m)$

2 *Ulbricht* (1969) uses the symbols α_s and β_s for the veratridine-modified Na channels

veratrine-treated muscle, the positive inotropic effect appears only during the course of several excitations above a critical frequency. This shows that the bioelectric and positive inotropic effect of veratrine are linked by a slowly responding accumulative process (Sect. 4.2.5). All the findings suggest that veratrine increases the force of contraction by increasing the intracellular Na concentration through its effect on the Na channels. An intracellular accumulation of Na ions is expected to activate Na-Ca exchange (Chap. 5) which could explain the positive inotropic effect.

4.2.3 Atrioventricular Node

There are no reports on the effect of ceveratrum alkaloids on the transmembrane electrical activity of cells of the atrioventricular node. Using extracellular recording electrodes, *Swain* and *McCarthy* (1957) studied the effect of ceveratrum alkaloids on atrioventricular conduction in the dog heart-lung preparation. Veratrine, its main constituents veratridine and cevadine, and protoveratrine (a mixture of the protoveratrines A and B) each slow atrioventricular conduction. In the case of veratrine this effect is particularly pronounced at high frequencies, while at low frequencies veratrine may even accelerate atrioventricular conduction. At high frequencies the effect of veratrine is characterized by alternation of atrioventricular conduction time. The spontaneous firing rate of the atrioventricular node recorded after elimination of the activity of the sinoatrial node is enhanced by protoveratrine.

4.2.4 Purkinje Fibres

Arbel et al. (1975) studied the effect of veratrine (Sigma) on the isolated ventricular conduction system of the dog heart with intracellular microelectrodes. They found that superfusion of Purkinje fibres in the right bundle branch with solution containing 0.1–1 μg/ml veratrine causes a slowing of repolarization that affects the plateau phase and terminal repolarization phase of the action potential. The latter effect may result in the appearance of a distinct after-depolarization. Higher veratrine concentrations induce oscillatory responses superimposed on the prolonged repolarization phase. The after-depolarization was followed in some experiments by a slight hyperpolarization. The prolonging effect on the repolarization phase is reversed by washout with veratrine-free solution. In ventricular muscle cells of the anterior papillary muscle dissected together with the bundle branch, the prolongation of the action potential by veratrine is less, both in absolute units and if related to the control duration, than that recorded simultaneously from Purkinje fibres.

The effect on repolarization is interpreted as resulting from the persistent activation of Na channels because it is inhibited by TTX (0.3–3 μmol/litre). *Arbel* et al. (1975) note that TTX abolished the effect of veratrine on repolarization at a time when the Purkinje fibre is still excitable, indicating that at least a major fraction of the normal Na channels that mediate the upstroke of the action potential is not blocked by that concentration of TTX. One might thus conclude that the Na channels activated by veratrine are more sensitive to TTX than normal Na channels. In this context it is of interest that even during the normal action potential of Purkinje fibres, a component of persistent and TTX-sensitive Na current was detected (*Attwell* et al. 1979); this explains why TTX has a marked shortening effect on the normal action potential of Purkinje fibres (*Coraboeuf* et al. 1979). It appears that, in the absence (*Coraboeuf* et al. 1979) as well as in the presence of veratrine (*Arbel* et al. 1975), the effect of TTX on the repolarization phase requires lower concentrations than its inhibitory effect on the rate of rise of the action potential. As pointed out by *Attwell* et al. (1979), such a differential sensitivity does not necessarily indicate that the Na channels activated during upstroke of the action potential and those activated during the plateau phase differ in their sensitivity to TTX. Rather, the blockade of the same small fraction of Na channels by TTX may result in a larger effect on action potential duration than on upstroke velocity, if the small fraction of the Na current blocked by TTX is a large fraction of the *net* inward membrane current flowing during the plateau phase. Thus, the different sensitivity to TTX of the two phases of the action potential might be due to the fact that the upstroke is almost entirely the result of net inward Na current through Na channels, whereas during the plateau a considerable fraction of the inward ionic current is balanced by outward K current. This consideration applies equally to the situation when the action potential is prolonged by Na channel-gate toxins. It is therefore not necessary to postulate that veratrine activates an additional population of Na channels differing by an abnormally high sensitivity to TTX from normal Na channels responsible for the upstroke of the action potential. In the frog node of Ranvier, normal and veratridine-modified Na channels were identical in their sensitivity to TTX (*Ulbricht* 1974).

Veratridine (0.1 μmol/litre) has recently been shown to increase the force of contraction of canine Purkinje fibres stimulated at 1 Hz (*Vassalle* and *Bhattacharyya* 1980). The positive inotropic effect, as well as the prolongation of the action potential observed in the same study, are antagonized by the local anaesthetics procaine or benzocaine.

4.2.5 Ventricular Muscle

Although the direct positive inotropic effect of ceveratrum alkaloids had already been discovered at the beginning of this century (see *Krayer* and *Acheson* 1946), analytical and quantitative studies of this effect were performed only in recent years (*Reiter* 1963; *Koch-Weser* 1966; *Horackova* and *Vassort* 1973, 1974; *Honerjäger* and *Reiter* 1975, 1977a; *Honerjäger* 1977, 1980). An understanding of the cardiac effects of these alkaloids was only possible after *Ulbricht* (1969) had shown that veratridine alters the Na channels of the excitable membrane. Detailed information on the effects of individual ceveratrum alkaloids on ventricular myocardium was obtained in the isolated isometrically contracting papillary muscle of the guinea pig (*Honerjäger* and *Reiter* 1975, 1977a; *Honerjäger* 1977, 1980).

In this section, various aspects of the positive inotropic effect are described including the concentration-effect relationships, the modifications of individual phases of the isometric contraction curve and the differentiation of the direct from a neurally mediated positive inotropic effect. Effects on transmembrane electrical activity are also described. Finally, the mechanism of positive inotropic action is discussed, as well as arguments suggesting that the positive inotropic effect is an indirect consequence of the modification of sarcolemmal Na channels leading to increased Na influx, intracellular Na accumulation and increased Na-Ca exchange.

Positive Inotropic Effect

The positive inotropic effect of ceveratrum alkaloids on the isometrically suspended guinea-pig papillary muscle results from an increase in the *rate* of force development, while the time to peak force is unaltered or slightly shortened (Figs. 2, 3). The term klinotropic was introduced to denote influences on the steepness of the contraction curve (*Bohnenkamp* 1922; *Reiter* 1972a). The positive inotropic effect of ceveratrum alkaloids is thus of the positive klinotropic type. With respect to their influence on the relaxation phase, two types of effect can be distinguished. Veratridine (Fig. 2), as well as cevadine (*Reiter* 1963; *Honerjäger* 1977), veracevine, zygadenine, and veratroylzygadenine (*Honerjäger* 1977) markedly prolong relaxation time and thereby total contraction time. In contrast, no major effect on total contraction time is observed under the influence of germitrine (Fig. 3), other germine di- and triesters or the protoveratrines A and B (*Honerjäger* 1977). This varied effect on the relaxation phase is probably due to the different modifications of the repolarization phase by these alkaloids. Furthermore, TTX inhibits the effect of veratridine on relaxation in addition to that on the repolarization phase suggesting a causal connection (see Fig. 9). A direct effect on the rate of Ca sequestration by sarcoplasmic reticulum thus seems to be ruled out.

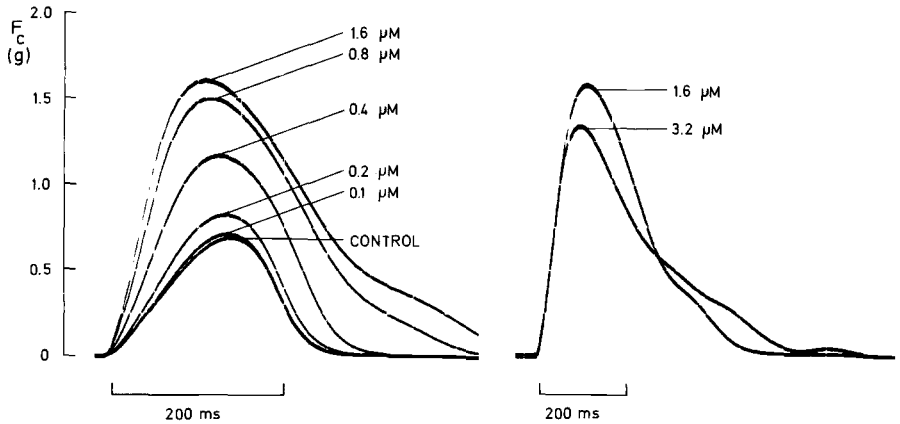


Fig. 2. The positive inotropic effect of veratridine. Isometric contraction records from an isolated guinea-pig papillary muscle contracting at 1 Hz and exposed to cumulatively increasing concentrations of veratridine. Note the prolongation of the relaxation phase. (From *Honerjäger* and *Reiter* 1975)

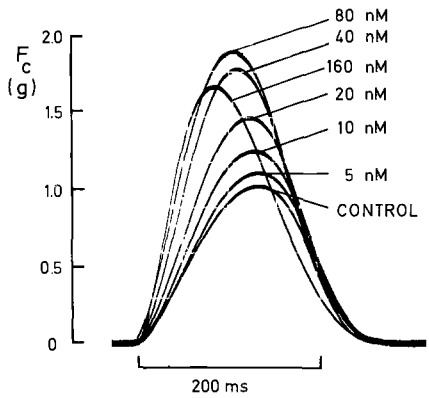


Fig. 3. The positive inotropic effect of germitrine. Isometric contraction records from an isolated guinea-pig papillary muscle contracting at 1 Hz and exposed to cumulatively increasing concentrations of germitrine. Note that this ceveratrum alkaloid does not markedly alter the duration of contraction. (From *Honerjäger* and *Reiter* 1977a, by permission of American Heart Association)

Indeed, veratridine (1 µmol/l to 1 mmol/l) is reportedly without effect on Ca uptake by sarcoplasmic reticulum isolated from rabbit skeletal muscle (*Johnson* and *Inesi* 1969).

The positive inotropic activity resides in the alkamine part of the ceveratrum alkaloids, but esterification increases the potency. The alkamines zygadenine and veracevine (each at 0.5 mmol/litre) produce a positive inotrpic effect, while germine (0.8 mmol/litre) and protoverine (5 mmol/litre) are inactive (*Honerjäger* 1977). Graded, steady-state positive inotropic effects can be obtained with different concentrations of ceveratrum alkaloids, suggesting a reversible binding reaction with the receptor for the positive inotropic effect. In fact, the positive inotropic effect of

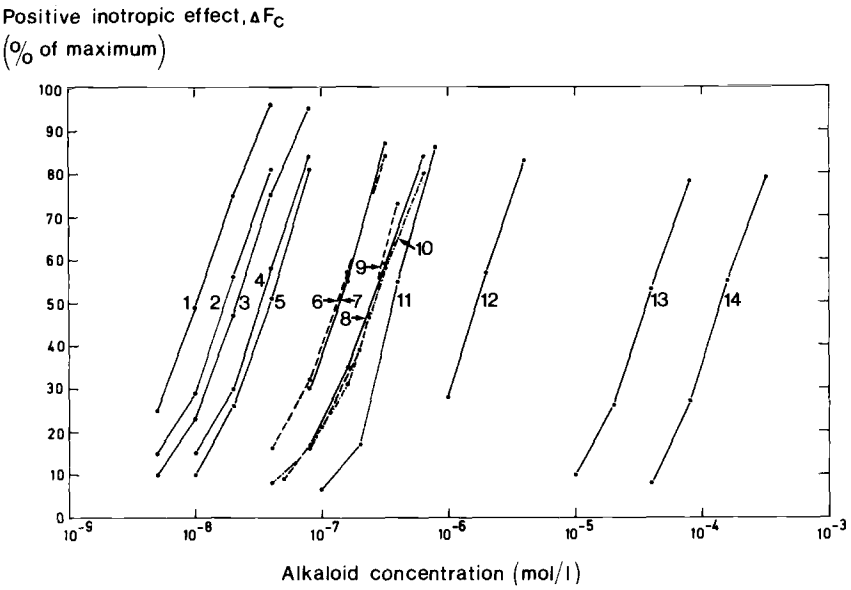


Fig. 4. Concentration-effect relationships for the positive inotropic effect of cevera-trum ester alkaloids: *Abscissa,* molar concentration of alkaloid, logarithmic scale; *ordinate,* positive inotropic effect expressed as percent of the maximum increase in force of contraction obtained with each alkaloid. Means of at least six guinea-pig papillary muscles for each alkaloid (SEM $< 7\%$).Germitetrine (1), protoveratrine A (2), germitrine (3), protoveratrine B (4), neogermitrine (5), desacetylgermitetrine (6), germerine (7), germbudine (8), veratroylzygadenine (9), neogermbudine (10), vera-tridine (11), cevadine (12), zygacine (13), and germine-3-acetate (14). (From *Honer-jäger* 1977)

veratridine disappears during washout with alkaloid-free medium, although only very slowly (*Honerjäger* and *Reiter* 1975). The concentration-depen-dence of the positive inotropic effect obtained with 14 different cevera-trum ester alkaloids is shown in Fig. 4. The absolute maximum increase in force of contraction, corrected for cross-sectional area of individual muscles, does not differ significantly among the various alkaloids. The concentration-effect relationships are nearly parallel and relatively steep. Each curve extends over approximately 1.5 logarithmic units. The con-centration-effect relationship for the increase of P_{Na} by veratridine is less steep and compatible with a reversible one-to-one binding reaction between veratridine molecules and the receptor at the Na channel (*Catte-rall* 1975). Presumably, the concentration-effect curve for the positive inotropic effect of ceveratrum alkaloids is limited by the capacity of the muscle to increase force development and not by the ability of cevera-trum alkaloids to increase sarcolemmal P_{Na} (*Honerjäger* and *Reiter* 1975, 1977a). The maximum positive inotropic effect of veratridine and

germitrine was found to be 68% and 81%, respectively, of that of the cardioactive steroid dihydro-ouabain tested on the same papillary muscle (*Honerjäger* and *Reiter* 1975, 1977a).

Like other depolarizing interventions, veratridine causes a release of noradrenaline from intracardiac adrenergic nerve endings (*Moss* et al. 1974). The release is mainly of the exocytotic type (*Thoa* et al. 1975; *Ross* and *Kelder* 1979). In addition, veratridine has a TTX-resistant, reserpine-like noradrenaline-depleting effect on the storage vesicles, but this effect does not normally lead to increased release of noradrenaline from the nerve ending (*Bönisch* et al. 1980). The positive inotropic effect of ceveratrum alkaloids, including veratridine, persists in muscles that have been depleted of noradrenaline by reserpine pretreatment (*Koch-Weser* 1966; *Honerjäger* and *Reiter* 1975, 1977a). This argues against the participation of a neural mechanism in the positive inotropic action under normal conditions (1 Hz contraction frequency). In contrast, concentrations of veratridine or cevadine exceeding those maximally effective at a frequency of 1 Hz increase the force of the rested-state contraction entirely by a neurally mediated mechanism (*Honerjäger* 1980). This positive inotropic effect is absent in the presence of a neuroactive, but not yet cardioactive concentration of TTX (100 nmol/litre) and in muscles from reserpine-pretreated animals. It is inhibited by 50 nmol/litre (−)propranolol, but not by the same concentration of the dextrorotatory enantiomer. The concentration-effect relationships for the direct and the neurally mediated positive inotropic effect of veratridine are illustrated in Fig. 5. While all these findings demonstrate that ceveratrum alkaloids affect isolated ventricular mammalian myocardium normally by a direct myotropic action, *Basseches* and *Bianchi* (1976) conclude, on the basis of their experiments on the perfused frog ventricle, that veratridine acts by enhancing transmitter release from cholinergic and adrenergic nerve fibres. The discrepancy may in part be due to the fact that *Basseches* and *Bianchi* (1976) used a field stimulus on their preparations which also excited both cholinergic and adrenergic nerve terminals. Under these conditions the frog ventricle may be more sensitive to the neural effects of veratridine.

Transmembrane Electrical Activity

Veratridine prolongs the repolarization phase of the action potential in guinea-pig papillary muscle (*Honerjäger* and *Reiter* 1975) and the effect of a relatively high concentration (2 μmol/litre) is illustrated in Fig. 6. For this experiment the papillary muscle was stimulated only once every 5 min which, because of the frequency-dependence of the inotropic action, prevents the positive inotropic effect of veratridine. Secondary superimposed effects on the action potential expected to result from

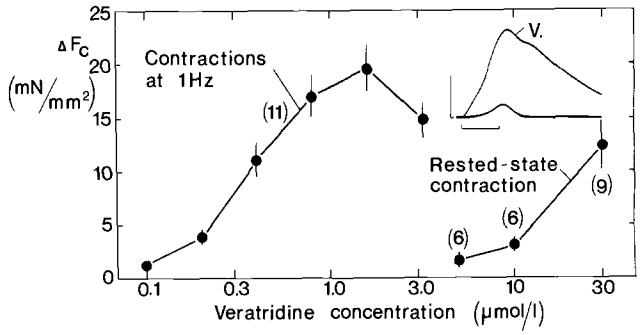


Fig. 5. Concentration-effect relationships for the direct *(left curve)* and neurally mediated *(right curve)* positive inotropic effect of veratridine on guinea-pig papillary muscle. The direct (reserpine-insensitive) effect was measured on muscles contracting at 1 Hz and exposed to cumulatively increasing concentrations. The presynaptic (reserpine-sensitive) effect was obtained on muscles that were incubated at rest with a single concentration of veratridine to determine its effect on the subsequent rested-state contraction. Number of muscles is given in parentheses. The *inset* shows a control rested-state contraction and a second one amplified by exposure of the resting muscle to 30 μmol/litre veratridine *(V)*; calibration: 5 mN (vertical), 0.2 s (horizontal). (From *Honerjäger* 1980)

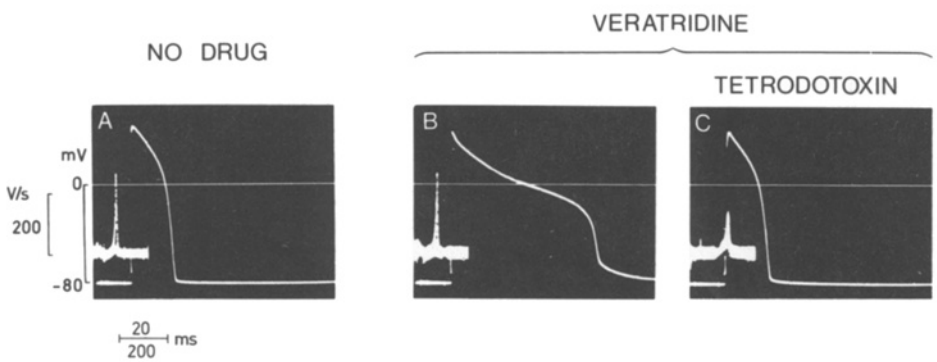


Fig. 6 A–C. Effect of veratridine on the transmembrane action potential of guinea-pig papillary muscle and blockade of the effect by TTX. Before **(A)**, 25 min after addition of 2 μmol/litre veratridine **(B)**, and 7 min after additonal application of TTX (10 μmol/litre) **(C)**. Continuous microelectrode impalement. Stimulation frequency was 0.2 min^{-1}. The broad trace is the differentiation of the upstroke of the action potential and was obtained at the faster sweep speed. (From *Honerjäger* 1977)

increased intracellular Ca, notably an abbreviation of the action potential (*Isenberg* 1975; *Bassingthwaighte* et al. 1976), are hence avoided. Veratridine does not affect the resting membrane potential or $\dot{V}_{max}$ (Fig. 6). The overshoot of the action potential is slightly reduced (by 4 mV). The plateau phase assumes an upward concave contour in contrast to the convex shape present under control conditions. The repolarization phase is

slowed particularly as it proceeds from +16 mV to −20 mV and from −60 mV to the resting potential level. Additional application of TTX (10 μmol/litre) completely restores the original action potential duration while reducing $\dot{V}_{max}$ by about 50% (Fig. 6C). The sensitivity of the veratridine effect to TTX strongly suggests that it is mediated by Na channels. As discussed in Sect. 4.2.4, the stronger effect of TTX on repolarization than on $\dot{V}_{max}$ does not argue against the identity of the Na channels responsible for the upstroke with those activated by veratridine during the plateau phase. A prolongation of the cardiac action potential may lead to an extracellular accumulation of K ions resulting in an after-depolarization (*Cleeman* and *Morad* 1976). In the presence of veratridine such an effect may play an additional role during the final phase of repolarization.

Reiter (1963) pointed out that the monoesteralkaloids veratridine and cevadine differ in their effect on the repolarization phase. Cevadine prolongs the plateau phase of the action potential whereas veratridine predominantly slows the subsequent phase of normally fast repolarization. This difference is reflected by the relaxation phase of the isometric contraction. Cevadine slows the initial part and veratridine delays the final part of relaxation (*Reiter* 1963).

The effect of the germine triester *germitrine* on action potential differs from that of veratridine in that only the very final phase of repolarization is prolonged, causing the appearance of an after-depolarization lasting longer than 1 min (*Honerjäger* and *Reiter* 1977a). As shown in Fig. 7, germitrine stops the repolarization process at a level about 6 mV positive to the resting potential. Thereafter the membrane depolarizes again slightly, to a maximum level about 9 mV positive to the resting potential, and then repolarizes very slowly. The original level of the resting potential is eventually reached after a slight hyperpolarization (Fig. 8). The after-depolarization produced by di- and triesters of germine, as well as by the two tetraesters of protoverine, protoveratrine A and protoveratrine B (Fig. 8) must be distinguished from the oscillatory after-potentials observed under conditions of Ca overload. The latter occur after complete repolarization and in an oscillatory manner: They are of shorter duration and not mediated by Na channels (Sect. 4.1.2). The germitrine-induced after-depolarization is completely and reversibly blocked by 10 μmol/litre TTX, suggesting that it results from a delayed activation of Na channels (*Honerjäger* and *Reiter* 1977a). The amplitude of the after-depolarization increases with the concentration of germitrine.

The demonstration of the germitrine effect requires a very low stimulation frequency (cf. Fig. 7). If a germitrine-treated muscle is stimulated repetitively at intervals shorter than the duration of the germitrine-induced after-depolarization, the resting potential shows a stepwise

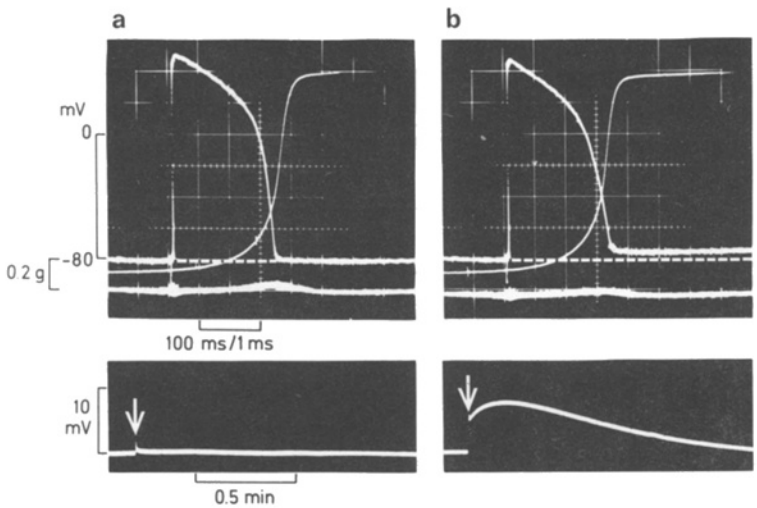


Fig. 7 a,b. Effect of germitrine on the transmembrane action potential and contraction of a guinea-pig papillary muscle stimulated once every 10 min. The *upper panels* show the action potential and isometric contraction at a slow sweep speed and the upstroke of the action potential at a fast sweep speed. The records shown in the *lower panels* were obtained simultaneously with the corresponding upper records on a second oscilloscope at higher gain and very slow sweep speed. They show the membrane potential before and during a period of 1.5 min after the action potential. The action potential causes the interruption *(arrow)* of these traces. Records were obtained before (**a**) and 74 min after (**b**) addition of 2 μmol/litre germitrine. Note incomplete repolarization (**b**, *upper panel*), which gives rise to an after-depolarization (**b**, *lower panel*). (From *Honerjäger* and *Reiter* 1977a, by permission of American Heart Association)

decrease (*Honerjäger* and *Reiter* 1977a). This phenomenon is analogous to the well-known "summation" of after-depolarizations observed if veratridine-treated nerve fibres are subjected to high-frequency stimulation (*Ulbricht* 1969). In the presence of high germitrine concentrations, the repetitive stimulation leads eventually to arrhythmic activity of the papillary muscle if the after-depolarization reaches the threshold to elicit an action potential. At 80 nmol/litre germitrine, the maximally effective inotropic and non-arrhythmic concentration at 1 Hz frequency, the summation of after-depolarizations results in a decrease of the resting potential by about 10 mV in the steady-state (*Honerjäger* and *Reiter* 1977a).

Mechanism of the Positive Inotropic Effect

Studies of the effects of veratridine (*Reiter* 1963; *Honerjäger* and *Reiter* 1975) and germitrine (*Honerjäger* and *Reiter* 1977a) revealed a close association of the bioelectric and inotropic effects in guinea-pig papillary muscle that strongly suggests a causal relationship. The results may be summarized as follows:

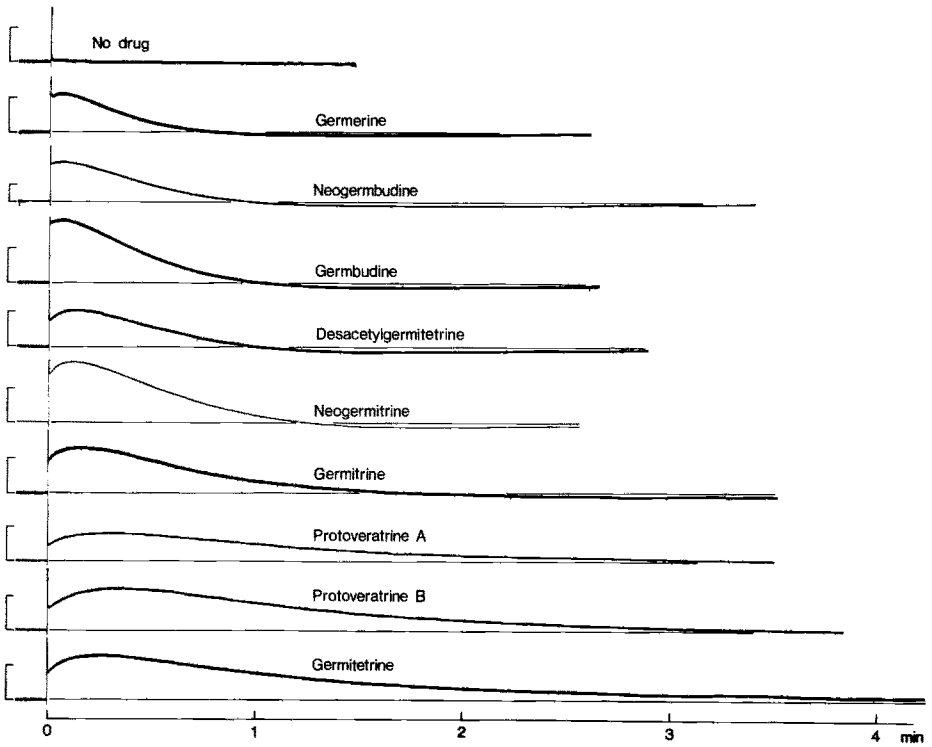


Fig. 8. After-depolarizations induced in guinea-pig papillary muscle by various cevegetatrum ester alkaloids. High-gain records of transmembrane potential immediately before and after the stimulation of an action potential. The action potential causes the interruption of the traces at the time marked 0. Experimental procedure as in Fig. 7. The following alkaloid concentrations were used (μmol/litre): germerine (4); neogermbudine (10); germbudine (8); desacetylgermitetrine (4); neogermitrine (2); germitrine (2); protoveratrine A (3); protoveratrine B (12); and germitetrine (4). Vertical calibration was 5 mV. (From *Honerjäger* 1977)

1. Inotropically effective concentrations of veratridine or germitrine also produce changes of the transmembrane electrical activity attributable to an increased P_{Na}.
2. TTX inhibits the bioelectric as well as the positive inotropic effect of veratridine or germitrine.
3. While the bioelectric and inotropic effects appear to occur simultaneously in repetitively stimulated preparations, a closer analysis reveals that the positive inotropic effect is in fact delayed with respect to the phase of increased P_{Na} produced by either veratridine (*Honerjäger* 1977) or germitrine (*Honerjäger* and *Reiter* 1977a) and decays slowly in the non-excited muscle.

4. As a consequence of the delayed and transient inotropic response to the alkaloid-induced P_{Na}, the positive inotropic effect does not manifest itself when contractions are separated by long rest intervals (e.g., 5 min). The inotropic effect appears and grows as the frequency is increased from 0.004 Hz to 0.5 Hz.
5. The positive inotropic response to the combination of a ceveratrum alkaloid with dihydro-ouabain is much larger than the sum of the responses to either drug alone. This synergism points to the importance of the intracellular Na concentration in mediating the positive inotropic effect of ceveratrum alkaloids. The extent of the alkaloid-induced increase in $[Na]_i$ must be enhanced by an additional inhibition of the Na pump.

TTX has proved a valuable tool in identifying the mechanism of the positive inotropic action of ceveratrum alkaloids and Na channel-gate toxins in general. The selective action of TTX in blocking Na channels of excitable membranes at an extracellular membrane site has been extensively documented (see *Narahashi* 1974). While suppressing I_{Na}, TTX does not significantly affect the slow inward current in frog atrial myocardium (*Tarr* 1971) or, at 47 μmol/litre, in dog ventricular myocardium (*Beeler* and *Reuter* 1970b). With regard to the analysis of changes in contractile activity it is important to note that TTX does not appear to penetrate through the plasmalemma as shown in studies on the squid giant axon (*Narahashi* et al. 1966; *Rojas* and *Rudy* 1976). Thus, TTX is unlikely to interfere directly with any intracellular process of excitation-contraction coupling or the contraction of the contractile proteins. TTX may affect myocardial force of contraction by two recognized mechanisms[3]. It abolishes the positive inotropic effect associated with field stimulation by blocking the excitability of intracardiac adrenergic nerve fibres (*Feinstein* and *Paimre* 1968). This effect requires very low concentrations (31–63 nmol/litre) that have virtually no effect on the Na channels of guinea-pig myocardial cells as judged by the upstroke of the action potential (*Baer* et al. 1976). Similarly low concentrations of TTX likewise abolish the presynaptic (or "indirect") positive inotropic effect of veratridine or cevadine (see p. 26). The second mechanism by which TTX affects myocardial force involves the blockade of sarcolemmal Na channels. At a frequency of 1 Hz, 10 μmol/litre TTX reduces $\dot{V}_{max}$ by about 50% and peak force of contraction by about 30% in noradrenaline-depleted guinea-pig papillary muscle (*Honerjäger* and *Reiter* 1975). This direct negative inotropic action has to be taken into account when TTX is used as an antagonist of positive inotropic agents.

3 The citrate buffer present in the commercial preparation of TTX does not cause a significant inotropic effect of its own when tested at a concentration corresponding to 15 μmol/litre TTX (*Honerjäger* and *Reiter* 1975)

The antagonistic effect of TTX against both the bioelectric and inotropic effects of veratridine is illustrated in Fig. 9. TTX completely and reversibly abolishes the positive inotropic effect of veratridine. The negative inotropic effect of TTX in the presence of veratridine is much larger than that observed in its absence, showing the specific nature of the antagonism. As Fig. 10 shows, TTX antagonizes the positive inotropic effect of ceveratrum alkaloids selectively. The effects of representatives of three other classes of inotropic agents, a cardioactive steroid, a catecholamine and a methylxanthine are not, or much less, inhibited by TTX. Interestingly, the positive inotropic effect of dihydro-ouabain, which also depends critically on Na influx (*Reiter* 1972a), is less sensitive to TTX than that of the ceveratrum alkaloids veratridine, cevadine, and germitrine. In the case of the steroid, the increase in intracellular Na concentration results from inhibition of the Na pump, and the extent of this increase is diminished if passive Na influx is inhibited by TTX. This might explain the slight antagonism observed (Fig. 10). TTX is expected to be more effective in reducing the alkaloid-induced Na influx than the normal Na influx, on which the inotropic action of the steroid may depend, for the following reasons: Firstly, the normal Na influx probably occurs through TTX-sensitive Na channels as well as through TTX-insensitive ion channels such as slow (Ca, Na) channels (*Reuter* and *Scholz* 1977). Secondly, the Na influx induced by Na channel-gate toxins during the repolarization phase is probably more strongly inhibited by TTX than the Na influx occurring during the upstroke, as suggested by the higher TTX sensitivity of the repolarization phase (Sect. 4.2.5).

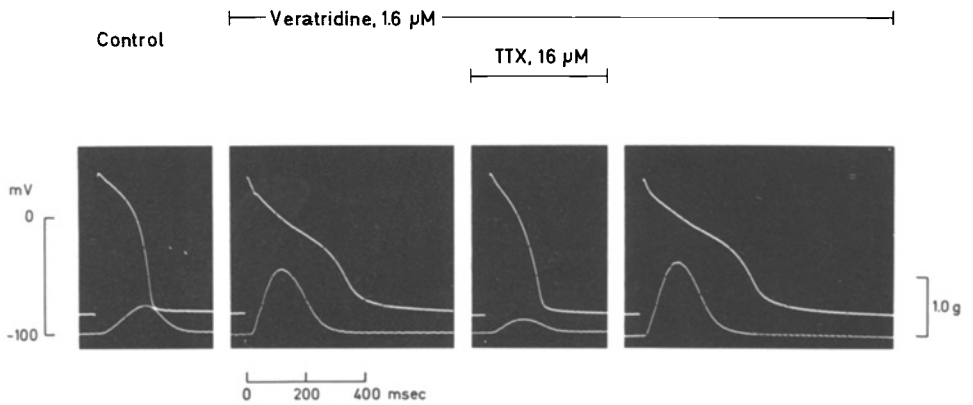


Fig. 9. Reversible inhibition of the effects of veratridine on action potential and contraction by TTX in guinea-pig papillary muscle. The records were obtained (from left to right) before drug application, 50 min after addition of veratridine (1.6 µmol/litre), 5 min after additonal application of TTX (16 µmol/litre), and after washout of TTX during maintained exposure to veratridine. Muscle was from a reserpine-pretreated animal. Contraction frequency was 1 Hz. (From *Honerjäger* and *Reiter* 1975)

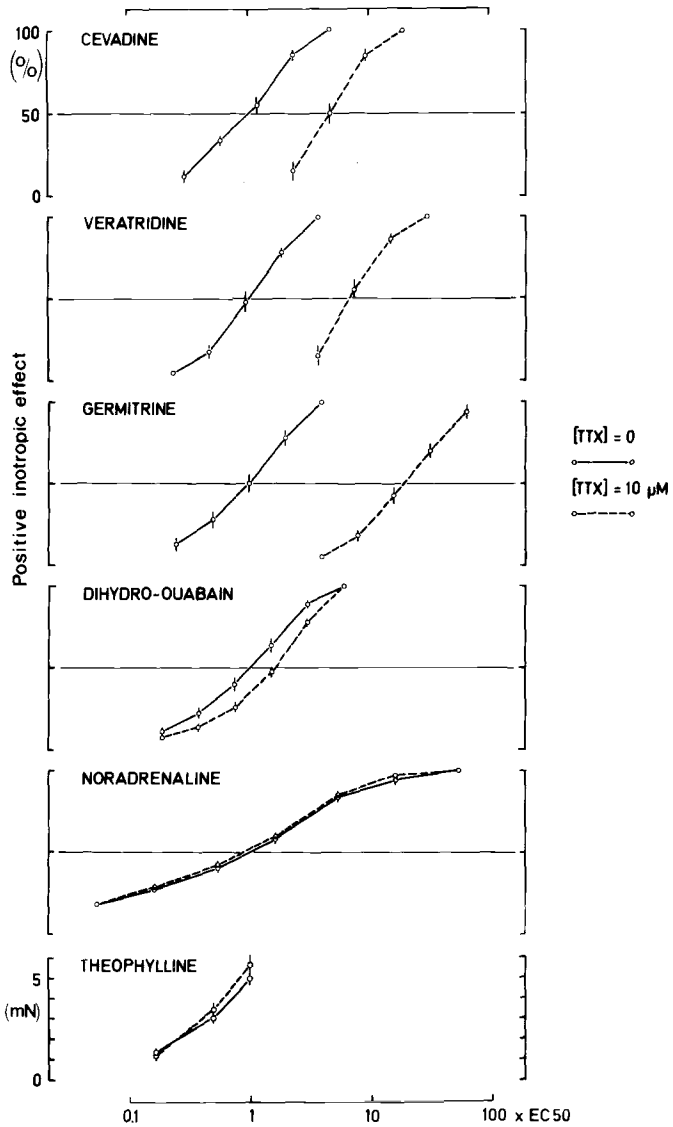


Fig. 10. Specificity of TTX as an antagonist of inotropic drugs. Concentration-effect relationships obtained in the absence and presence of TTX (10 µmol/litre) by cumulative drug additions. *Ordinate:* positive inotropic effect expressed as percent of the maximum effect obtainable under each condition, except in the case of theophylline where the absolute increase of force of contraction is given; *abscissa:* drug concentration in logarithmic scale expressed in units of half-maximally effective concentrations in the absence of TTX. Means ± SEM *(vertical bars)* of at least six guinea-pig papillary muscles. For the ceveratrum alkaloids (cevadine, veratridine, and germitrine) control and TTX experiments were done on separate muscles. Each of the other drugs was tested both in the absence and presence of TTX on the same muscle. Contraction frequency was 1 Hz. (From *Honerjäger* 1977)

The inhibitory effect of 10 μmol/litre TTX is fully surmountable by increases in the concentration of the ceveratrum alkaloids (Fig. 10). This should not be taken as evidence for a competitive type of antagonism between the alkaloids and TTX. The antagonism between Na channel-gate toxins and TTX at the level of Na channels is non-competitive (Sect. 3.10.1). The inotropically effective range of alkaloid concentrations probably modifies only a small fraction of Na channels, and this fraction can still be affected by the gate toxins at a TTX concentration (10 μmol/litre) that blocks only about 50% of the Na channels (*Honerjäger* and *Reiter* 1975).

Although the inhibition of veratridine effects by TTX suggests a causal relationship between the bioelectric and the inotropic effect of veratridine, it remains uncertain whether veratridine increases force of contraction by increasing Na influx or by prolonging the repolarization phase (thereby indirectly prolonging the phase of Ca influx through slow channels). However, the prolongation of the action potential, under the conditions of Fig. 9, is restricted to negative levels of membrane potential where a prolongation of repolarization per se produces little positive inotropic effect (*Wood* et al. 1969). In the case of germitrine the positive inotropic effect is even associated with a marked shortening of the action potential duration, in addition to the reduction of the resting membrane potential reflecting the increased P_{Na} (*Honerjäger* and *Reiter* 1977a). This rules out a secondary effect on I_{si} as a cause of the positive inotropic effect for this ceveratrum alkaloid. The strongest evidence in support of the hypothesis that ceveratrum alkaloids act by increasing inward Na current is provided by the voltage-clamp experiments of *Horackova* and *Vassort* (1973, 1974) on frog atrial muscle (Sect. 4.2.2).

The germitrine-induced after-depolarization is particularly suitable for a study of the temporal relationship between the phase of increased P_{Na} and the positive inotropic effect. By choosing an appropriate alkaloid concentration, the amplitude of the after-depolarization can be adjusted to a level which is well below the activation threshold for I_{si} (-40 mV; *New* and *Trautwein* 1972) and which leaves the muscle electrically excitable throughout the course of the after-depolarization. Test contractions elicited at various times during or after the alkaloid-induced after-depolarization (Fig. 11) reveal a phase during which the ability of the muscle to develop force is increased. This positive inotropic influence reaches its maximum 1 min after the conditioning stimulus and thereafter decays with a half-life of 2.5 min. In contrast, the after-depolarization attains a peak value about 10 s after the conditioning action potential and decays with a half-life of 32 s. Thus the phase of increased P_{Na} clearly precedes the phase of increased contractility. This is compatible with a role of the intracellular Na concentration in mediating the positive inotropic effect.

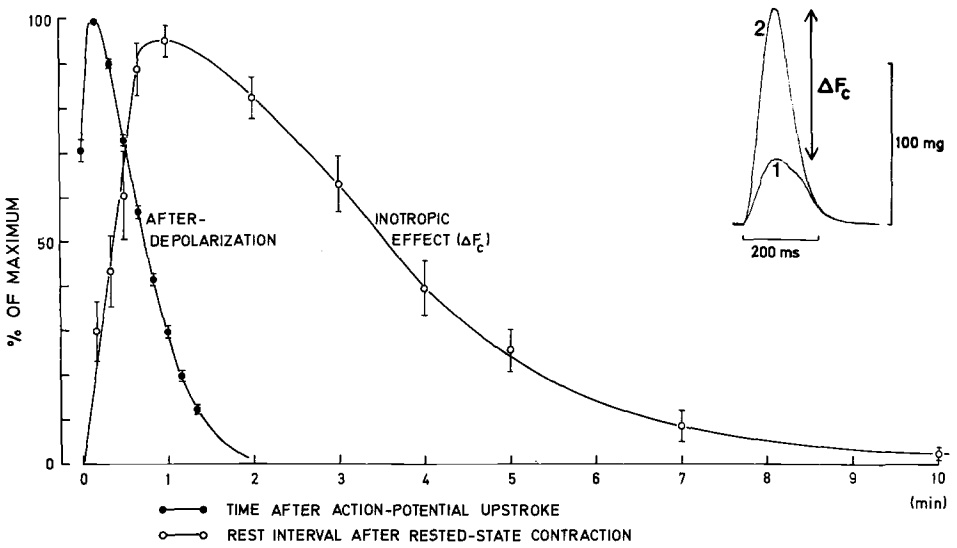


Fig. 11. Temporal relationship between the germitrine-induced after-depolarization and the germitrine-induced positive inotropic effect as determined by single test contractions during and after the after-depolarization. *Ordinate:* membrane potential during the after-depolarization which followed an action potential elicited at 0 time (100% = 8.1 ± 0.4 mV) (●) and peak force of test contraction minus peak force of prior rested-state contraction (100% = 79 ± 16 mg) (○). The time-course of the after-depolarization is shown by means ± SEM of 27–29 records obtained from six muscles incubated with 2 μmol/litre germitrine for at least 1 h. The positive inotropic effect (means ± SEM of six to eight muscles) was determined in different muscles. *Inset* shows enhancement of test contraction (trace 2) elicited 1 min after the conditioning rested-state contraction (trace 1), i.e. during the falling phase of the after-depolarization. (From *Honerjäger* and *Reiter* 1977a, by permission of American Heart Association)

$[Na]_i$ will continue to rise during the period of increased P_{Na} and subsequently decline owing to the activity of the Na pump. Direct verification of this point by the use of an intracellular Na-sensitive micro-electrode would be of great interest.

It is generally accepted that the contraction of cardiac cells is directly linked to the increase in free Ca concentration of the myoplasm. An increased intracellular Na concentration can mediate a positive inotropic effect indirectly by increasing the transsarcolemmal influx of Ca ions, thereby loading cellular Ca stores that release Ca during contraction. If Na-Ca exchange is enhanced by the depolarization produced by the action potential (Sect. 5.3.4; *Mullins* 1979), an increased level of $[Na]_i$ may not only increase the steady influx of Ca ions but especially that occurring during the action potential. The evidence suggesting the existence of a Na-Ca exchange mechanism in the plasmalemma is discussed in Chap. 5.

Few attempts have been made to rule out alternative mechanisms of the positive inotropic effect by direct biochemical experiments. The activity of Na, K-ATPase isolated from guinea-pig hearts is not affected by concentrations of ceveratrum alkaloids (veratridine, cevadine, protoveratrine A and B, germitetrine, desacetylgermitetrine) that are much higher than those causing a maximum positive inotropic effect on guinea-pig papillary muscle (*Portius* and *Repke* 1964). Veratridine, cevadine, and protoveratrine lack an inhibitory effect on the active Na-K transport of erythrocytes (*Kahn* and *Acheson* 1955). *Sperelakis* and *Pappano* (1969) noted that veratrine did not significantly change the internal K and Na content of intact embryonic chick hearts during 15 min, which argues against inhibition of the Na pump. There is apparently no report on whether inotropically effective concentrations of ceveratrum alkaloids influence the concentration of cyclic nucleotides in myocardial cells. However, it should be noted that many functional characteristics of the inotropic action of ceveratrum alkaloids (and other Na channel-gate toxins) differ clearly from those associated with agents thought to act by increasing the cellular content of cyclic adenosine 3′,5′-monophosphate (cAMP) (Sect. 5.1).

4.2.6 Cardiac Cells in Culture

During the embryonic development of chick and rat hearts, $\dot{V}_{max}$ of the cardiac action potential and the susceptibility of action potential and contraction to TTX were shown to increase progressively (*Bernard* and *Gargouil* 1968; *Ishima* 1968; *Shigenobu* and *Sperelakis* 1971; *Sperelakis* and *Shigenobu* 1972; *Pappano* 1972; *McDonald* et al. 1972; *DeHaan* et al. 1975; *McDonald* and *Sachs* 1975; *Iijima* and *Pappano* 1979). Thus, in the early embryonic stage (2–4 days in ovo), cardiac cells seem to lack fast Na channels. The action potential is mainly due to the opening of a slow Na channel insensitive to TTX (*Sperelakis* and *Shigenobu* 1972). Cardiac cells cultured in the form of monolayers invariably display TTX-insensitive action potentials even if the cells are taken from embryos older than 5 days. Cultured in aggregates, they generally retain the TTX sensitivity of the embryonic heart from which they were derived (*McDonald* et al. 1972; *McLean* and *Sperelakis* 1976).

Sperelakis and *Pappano* (1969) investigated the effects of veratrine and veratridine on ventricular myoblasts obtained from 7–15-day chick embryos, but grown in monolayers. Veratridine (1.5–15 μmol/litre) depolarizes the cells within a few minutes to membrane potentials of about −12 mV, and action potentials and beating cease. Before the depolarization begins, the repolarization phase of the action potential is prolonged by veratridine. TTX (63 μmol/litre), although without effect

on action potential magnitude or $\dot{V}_{max}$, prevents the depolarization and prolongation of the action potential by veratridine. The depolarization by veratridine also occurs in Na-free Li solution. Veratridine reduces the membrane resistance. This is not prevented by TTX and is attributed to an increased K permeability. In 3- and 16-day aggregates of chick embryonic heart cells, veratridine produces depolarization and blocks the electrical and mechanical activities at concentrations exceeding 10 μmol/litre (*Romey* et al. 1980). *Romey* et al. (1980) suggest that, like other Na channel-gate toxins, veratridine reveals Na channels that are normally in a silent form in monolayers or 3-day aggregates.

The activation of Na channels by veratridine leads to an increase of Na influx into the myoblasts as determined with ^{22}Na (*Fosset* et al. 1977). The effect is inhibited by TTX, the half-maximally effective concentration being 6.6 nmol/litre at a veratridine concentration of 22 μmol/litre (*Fosset* et al. 1977). This degree of TTX sensitivity is similar to that of $\dot{V}_{max}$ in cells at later stages of embryonic development (*McDonald* et al. 1972; *Romey* et al. 1980), but much higher than that of Na channels in adult guinea-pig ventricular myocardium (*Baer* et al. 1976; see also Fig. 6). Veratridine increases the influx of Ca ions in addition to that of Na ions (Sect. 5.3.2). Protoveratrine B also increases the Na uptake by cardiac cells of the chick embryo (*Couraud* et al. 1976).

4.3 Batrachotoxin

Batrachotoxin belongs to the most toxic substances known. Its lethal effect on mammals, which occurs after parenteral administration of less than 1 μg/kg or 1.86 nmol/kg, appears to be mainly due to its cardiac arrhythmogenic action (*Daly* and *Witkop* 1971). A study on anaesthetized rabbits showed that batrachotoxin produces a variety of ventricular arrythmias, in addition to complete block of atrioventricular conduction (*Kayaalp* et al. 1970). Predominant effects are the activation of multifocal ventricular ectopic beats interrupted by transient ventricular fibrillation or tachycardia. The terminal event is ventricular fibrillation.

4.3.1 Purkinje Fibres

In spontaneously active Purkinje fibres isolated from the dog heart, the initial effect of batrachotoxin on the action potential is a prolongation of the final phase of repolarization (*Hogan* and *Albuquerque* 1971). It occurs at the lowest tested concentration of 1.9 nmol/litre at a time when there are no changes in the maximum diastolic membrane potential, $\dot{V}_{max}$, overshoot of the action potential, or rate of spontaneous diastolic depolarization. With increasing time of exposure to the toxin, the delay

of repolarization progresses until the membrane fails to repolarize completely during the diastolic interval. At this stage the upstroke velocity and overshoot of the action potential are depressed, as expected from the inactivating influence of depolarization. The membrane potential of fibres depolarized by batrachotoxin is restored if the external Na concentration is reduced to 1 mmol/litre. At a concentration of 4.7 μmol/litre, TTX prevents the effect of batrachotoxin (1.9 nmol/litre) on the repolarization phase for as long as 30 min. These results clearly indicate the involvement of Na channels in the effect of batrachotoxin on the Purkinje fibre membrane. *Hogan* and *Albuquerque* (1971) point out that "TTX did not block the action potential of the Purkinje fiber in a concentration which antagonized BTX [batrachotoxin] action". According to these authors, "resting sodium channels are opened by BTX" and "resting sodium channels in the electrogenic membrane may be functionally different from the sodium channels for membrane excitation". The TTX concentration used (4.7 μmol/litre) causes a partial inhibition of $\dot{V}_{max}$ in dog Purkinje fibres (approx. 50%, *Coraboeuf* et al. 1979). That this concentration causes complete inhibition of the effect of batrachotoxin, as opposed to partial inhibition of $\dot{V}_{max}$, does not argue against the identity of the Na channels involved in generating the action potential with those modified by batrachotoxin, as discussed in connection with the effect of veratrine on dog Purkinje fibres (Sect. 4.2.4). *Hogan* and *Albuquerque* (1971) state that "the same sequence of alterations produced by BTX on the Purkinje fiber membrane was observed whether or not the tissue was being driven". Since their preparations were reportedly spontaneously active, this does not rule out a dependency of the effect of batrachotoxin on electrical activity. Such a dependency was reported to exist in the eel electroplaque (*Bartels-Bernal* et al. 1977), frog node of Ranvier (Sect. 3.3), and guinea-pig papillary muscle (Sect. 4.3.2).

4.3.2 Ventricular Muscle

Similar to its effect on Purkinje fibres, batrachotoxin prolongs the repolarization phase of the action potential in guinea-pig muscle, and this effect is inhibited by TTX (*Honerjäger* and *Reiter* 1977b). The results indicate that batrachotoxin causes its effect on the ventricular action potential by selectively prolonging the open state of Na channels. At low concentrations of the alkaloid (0.75–60 nmol/litre), the effect on repolarization occurs only after a series of conditioning action potentials. The first action potential after addition of batrachotoxin is unaltered even after prolonged exposure of the resting muscle to the toxin. During repetitive stimulation, the effect on action potential duration develops at a progressively increasing rate and eventually gives rise to spontaneous depolariza-

tions arising from the prolonged repolarization phase. For a given effect, measured in terms of the prolongation of the action potential or the first appearance of a coupled extrasystole, the product of batrachotoxin concentration and number of preceding action potentials during exposure to that concentration of batrachotoxin is virtually constant. The effect thus fits the quantitative requirements of an irreversible one-to-one binding reaction of batrachotoxin molecules with sarcolemmal receptors that are accessible only during the action potential. As in the frog node of Ranvier (Sect. 3.3), the binding of batrachotoxin seems to require specifically the opening of Na channels. Prolonged depolarization by exposure to a high-K solution does not lead to the expression of the batrachotoxin effect in guinea-pig papillary muscle. Removal of batrachotoxin from the bathing solution during continued stimulation of the muscle causes the effect to decrease gradually over several hours, indicating a very slow dissociation of batrachotoxin. The dissociation of batrachotoxin (as manifested by the disappearance of its effect on the repolarization phase) does not require activity and can thus be demonstrated, even in the presence of toxin, simply by resting the muscle.

Concomitantly with the prolongation of the action potential, batrachotoxin produces a positive klinotropic and positive inotropic effect and prolongs relaxation time in guinea-pig papillary muscle (*Honerjäger* and *Reiter* 1977b). These effects persist in preparations from reserpine-pretreated animals, and are prevented by the presence of TTX (10 μmol/litre). The sensitivity to TTX suggests that the positive inotropic effect results from the increased Na influx induced by batrachotoxin. A reserpine-resistant, TTX-sensitive positive inotropic effect of batrachotoxin is also observed in the isolated cat papillary muscle (*Shotzberger* et al. 1976). In guinea-pig papillary muscle (*Honerjäger* and *Reiter* 1977b), the rested-state contraction elicited during incubation of the resting muscle with high concentrations of batrachotoxin (0.6–1.2 μmol/litre) is increased in amplitude and rate of rise. This inotropic effect is absent in muscles from reserpine-pretreated guinea-pigs, pointing to the involvement of endogenous noradrenaline as in the case of high concentrations of veratridine or cevadine (Sect. 4.2.5).

4.4 Aconitine

4.4.1 Sinoatrial Node and Atrium

In a preparation consisting of the sinoatrial node and extranodal tissue of the rabbit heart, aconitine (1 μg/ml) produces a bigeminal rythm, followed by flutter and irregular activity (*Matsumura* and *Takaori* 1959b).

The intracellular records demonstrate that both nodal and extranodal fibres participate in the abnormal rhythm.

4.4.2 Purkinje Fibres

Aconitine produces very similar effects on Purkinje fibres isolated from dog (*Schmidt* 1960), calf or sheep hearts (*Heistracher* and *Pillat* 1962). Aconitine increases the rate of spontaneous depolarization thus causing a positive chronotropic effect on isolated Purkinje fibres. At this stage of action, parameters of the transmembrane action potential are not altered. Subsequently, aconitine selectively slows the terminal part of repolarization (at about -60 mV) which results in the appearance of after-depolarization. The duration of the after-depolarization increases from beat to beat until premature action potentials arise from the prolonged repolarization phase. Following a stage of bigeminy and trigeminy, a flutter-like activity results and, finally, the preparations are arrested at a reduced membrane potential (-55 to -45 mV). Decreasing the extracellular Na concentration to 10% of the normal level causes an immediate, though transient, interruption of the aconitine-induced spontaneous activity (*Schmidt* 1960).

Peper and *Trautwein* (1967) analysed the effect of aconitine on sheep Purkinje fibres with a voltage-clamp technique. In the presence of aconitine (1 μg/ml) the current-voltage relation, obtained by changing the membrane potential at constant and relatively slow rates, shows an abnormal inward current with a maximum near -60 mV. Application of TTX (63 μmol/litre) blocks this current as well as the normal excitatory Na current. In Na-free solution, aconitine fails to affect the current-voltage relation. These findings suggest that aconitine abolished the inactivation gate in a fraction of Na channels, thus causing a persistently activated component of P_{Na}.

4.3.3 Ventricular Muscle

Matsuda et al. (1959) describe the effects of amorphous aconitine (Merck) and of the purified alkaloids aconitine and mesaconitine on isolated right ventricular tissue of the dog heart. No essential difference was noted among the three alkaloidal preparations. A few minutes after drug administration (0.01–0.1 μg/ml), the muscle suddenly undergoes a flutter-like, automatic and rapid excitation. Non-stimulated, resting preparations do not become spontaneously active if aconitine is added, and aconitine does not affect the resting transmembrane potential in these fibres. The automatic activity is shown to result from one of two mechanisms. Most frequently, the first visible effect is a decrease of the resting potential, followed by oscillatory potential changes following each action potential

and superimposed on the decreased level of resting potential. *Schmidt* (1960), in his study on dog ventricular trabeculae, reported no oscillatory after-potentials under the influence of aconitine, but only a slowing of the final repolarization phase, which then led to premature action potentials. This mechanism of arrhythmogenesis was observed less frequently by *Matsuda* et al. (1959).

Heistracher and *Pillat* (1962) report the effects of aconitine (0.05–1.0 μg/ml) on transmembrane potentials of the isolated cat papillary muscle. The initial electrophysiologic effect appears to be a prolongation of the repolarization phase near its end (after-depolarization). The slowing of repolarization is less abrupt and less pronounced than that observed in dog ventricular muscle (*Schmidt* 1960). The after-depolarization may escape detection if the arrhythmogenic effect of aconitine develops rapidly. In the second stage of aconitine action, extrasystoles appear in the form of bigeminy or trigeminy. The premature action potentials seem to arise from the aconitine-induced after-depolarization. Subsequently, the papillary muscles show sustained high-frequency activity. The phase of automatic activity is followed by the termination of activity owing to a reduction of the resting membrane potential to about −55 mV in right ventricular muscle isolated from the rabbit heart (*Matsumura* and *Takaori* 1959a). An as yet unexplained effect of aconitine is the appearance of spike-like depolarizations arising from the normal resting potential and having an amplitude of up to 40 mV (*Heistracher* and *Pillat* 1962).

In the isolated cat papillary muscle, the time to development of aconitine-induced automaticity is inversely related to aconitine concentration (0.25–1 μg/ml), diastolic tension (60%–140% or peak tension) and stimulation frequency (1–4 Hz) during the preautomatic period (*Tanz* et al. 1973). As also noted by earlier investigators, the aconitine arrhythmia is not reversed by repeated washing of the preparations. The cumulative effect of repetitive stimulation may indicate that myocardial Na channels, like neural Na channels (*Mozhayeva* et al. 1977), interact with aconitine only when they are in the open configuration. The aconitine-induced arrhythmia is rapidly abolished by 3 μmol/litre TTX (*Tanz* 1974).

Although aconitine clearly induces a long-lasting activation of Na channels in Purkinje fibres (*Peper* and *Trautwein* 1967) and probably also in ventricular muscle, there is apparently no evidence that aconitine produces a positive inotropic effect. In this respect, aconitine differs from all other cardioactive Na channel-gate toxins reviewed in this chapter. It may seem questionable therefore whether an increase in sarcolemmal Na permeability is strictly linked to positive inotropic action. One possible explanation for the failure of aconitine to produce a positive inotropic effect under the conditions used by previous investigators (e.g., Fig. 1 in *Tanz* et al. 1973) is that the transition to the state of extrasystoles

and spontaneous activity occurs without a sufficiently long-lasting phase during which aconitine increases the Na influx associated with each action potential, but does not yet produce extrasystoles. In fact, while the stage of action characterized by the prolonged repolarization phase is easily demonstrated with many other Na channel-gate toxins, this has proven difficult with aconitine (*Heistracher* and *Pillat* 1962). A careful analysis of the electrophysiologic and inotropic effect of aconitine in the prearrhythmic phase of action is clearly necessary to settle this point. We have recently observed that the arrhythmogenic effect of aconitine (0.5–1.0 μmol/litre) is preceded by a small, but distinct positive klinotropic and inotropic effect in guinea-pig papillary muscle (*P. Honerjäger* and *A. Meissner,* unpublished work).

4.5 Grayanotoxins

4.5.1 Sinoatrial Node

In the isolated sinoatrial node preparation of the rabbit heart, grayanotoxin I (0.1 mmol/litre) decreases the maximum diastolic potential, increases the rate of spontaneous diastolic depolarization, thereby causing an increase in firing frequency and, finally, inhibits the action potentials (*Seyama* 1978). These effects are fully reversible by washing with toxin-free medium. TTX (10 μmol/litre) abolishes all effects on the transmembrane potentials, indicating that grayanotoxin I activates Na channels in the sinoatrial node. Thus, although this cell type is characterized by TTX-resistant electrical activity, the presence of Na channels can be demonstrated by pharmacological (*Kreitner* 1975) or electrical hyperpolarization (*Noma* et al. 1977) or by applying the Na channel-gate toxin grayanotoxin I (*Seyama* 1978).

4.5.2 Atrium

In resting right atrial strips obtained from the rabbit heart, grayanotoxin I (0.1 mmol/litre) produces a marked depolarization (by 26 mV), which is reversed by the withdrawal of external Na ions or the application of 10 μmol/litre TTX (*Seyama* 1978). A less-pronounced depolarization is observed with lower concentrations of grayanotoxin I or α-dihydro-grayanotoxin II applied to isolated and stimulated left atria of the guinea-pig (*Akera* et al. 1976; *Ku* et al. 1977). The addition of TTX (0.8 μmol/litre) reverses the depolarization produced by grayanotoxin I (0.5 μmol/litre) and partially restores the amplitude of the action potential, which is reduced by grayanotoxin I owing to the reduced resting potential (*Akera* et al. 1976). Thus grayanotoxin I presumably induces a persistent activa-

tion of Na channels resulting in depolarization of the resting membrane. Concentrations of grayanotoxin I that alter the membrane potential also cause a positive klinotropic and positive inotropic effect under isometric conditions (*Akera* et al. 1976). This effect is inhibited by TTX (0.8 μmol/litre). The racemic mixture of propranolol (10 μmol/litre) also inhibits the positive inotropic effect of both grayanotoxin I and α-dihydro-grayanotoxin II (*Ku* et al. 1977). These authors state "that a portion of the effects of grayanotoxins on isometric contractile force may be mediated by a beta-adrenergic mechanism". However, the Na channel blocking action of propranolol (*Morales-Aguillera* and *Vaughan Williams* 1965) might be responsible for this antagonism. At 5 μmol/litre, grayanotoxin I produces arrhythmia in isolated guinea-pig left atria (*Akera* et al. 1976). The grayanotoxins enhance the ouabain-sensitive uptake of the potassium analog rubidium by guinea-pig ventricular slices, which is interpreted as a stimulation of the Na-K pump secondary to the toxin-induced increase of Na influx (*Ku* et al. 1977). The grayanotoxins do not affect Na, K-ATPase isolated from rat brain or guinea-pig heart (*Akera* et al. 1976; *Ku* et al. 1977).

4.5.3 Atrioventricular Node

There are no reports on the effect of grayanotoxins on the transmembrane electrical activity of cells of the atrioventricular node. In the dog heart-lung preparation, examined with extracellular electrodes, grayanotoxin I (andromedotoxin) prolongs the atrioventricular conduction time and increases the spontaneous firing rate of the atrioventricular node if the faster rhythm of the sinoatrial node is suppressed (*Swain* and *McCarthy* 1957).

4.5.4 Purkinje Fibres

Conduction through the Purkinje system is reported to be slowed by grayanotoxin I (andromedotoxin) in the dog heart (*Swain* and *McCarthy* 1957). This occurs at a time when ventricular myocardial conduction is not affected. Possibly, the effect on Purkinje fibre conduction is related to a depolarization as observed in atrila cells (Sect. 4.5.2).

4.5.5 Ventricular Muscle

Hotta et al. (1980) studied 18 structurally related grayanotoxins which produced a concentration-dependent reversible positive inotropic effect on isolated guinea-pig papillary muscle.

4.6 Scorpion Toxins

4.6.1 Isolated Mammalian Heart

Corrado et al. (1968) investigated the effects of crude dried venom of the Brazilian scorpion *Tityus serruluatus* on the isolated guinea-pig heart. In final concentrations of approximately 1 μg/ml, the venom produces a short-lasting bradycardia followed by an increase of force and frequency of contractions. The effects are reversible and reproducible in the same preparation. The bradycardia is blocked by atropine and potentiated by neostigmine. The positive chronotropic and inotrpic action is abolished by propranolol or bretylium. It is absent in hearts of reserpine-pretreated guinea pigs. Hexamethonium neither affects the bradycardia nor the tachycardia in doses which abolish the nicotine-induced bradycardia and positive inotropic effect. Hence the venom probably acts on the postganglionic nerve terminals to cause the release of acetylcholine and noradrenaline. *Langer* et al. (1975) have shown that a purified toxin from *T. serrulatus* increases the release of ^{3}H-noradrenaline from guinea-pig atria. Less extensive experiments were performed by other investigators with venoms of *Leiurus quinquestriatus, Buthus minax, B. occitanus,* and *Androctonus australis* HECTOR using the isolated heart of rabbits or guinea pigs. Similar to *Tityus* scorpion venom, the other scropion venoms seem to alter cardiac function mainly through the release of neurotransmitters from intracardiac nerve fibres (*Zlotkin* et al. 1978).

Coraboeuf et al. (1975) used the isolated rat heart to study the effects of pure $ScTX_{II}$ on contraction and transmembrane electrical activity (at 24°–25°C). $ScTX_{II}$ produces a reserpine-resistant positive inotropic effect that is associated with an increase in the height of the plateau of the ventricular action potential and a prolongation of the repolarization phase. The effect on the action potential persists for hours following a single injection of toxin into the coronary arteries. Resting potential and $\dot{V}_{max}$ are only slightly altered. The lengthening of the action potential produced by $ScTX_{II}$ is greatly diminished by injections of TTX, Ca-rich medium or procaine, and it is prevented during perfusion with low-Na solution. The atrial action potential is also prolonged by $ScTX_{II}$. According to *Coraboeuf* et al. (1975), $ScTX_{II}$ increases plateau height by slowing down the inactivation of Na channels or by inducing an incomplete Na inactivation. The effect on the action potential might indirectly favour the penetration of Ca through the slow channel because of a stronger and longer-lasting activation of the potential- and time-dependent slow inward Ca current, and this could explain the positive inotropic effect. The authors do not discuss the possibility of increased Na-Ca exchange.

Confirming earlier investigations, *Coraboeuf* et al. (1975) find that $ScTX_{II}$ produces a positive inotropic effect also on isolated guinea-pig and rabbit hearts, but that this effect is absent in preparations obtained from reserpine-pretreated animals. Guinea-pig and rabbit ventricular action potentials are insensitive to the scropion toxin in doses that are markedly effective in the rat heart. As pointed out by *Coraboeuf* et al. (1975), this suggests the existence of species differences in the sensitivity of myocardial Na channels to scorpion toxin.

4.6.2 Cardiac Cells in Culture

$ScTX_{II}$ has marked effects on the mechanical and electrical activity of nerve-free myoblast cultures derived from the chick embryonic heart (*Fayet* et al. 1974; *Bernard* and *Couraud* 1979). $ScTX_{II}$ increases the frequency of the spontaneous contractions, and this effect is followed by cell fibrillation and contracture (*Fayet* et al. 1974). The bioelectric cause of these phenomena was elucidated by *Bernard* and *Couraud* (1979). They used cells from 11-day-old chick embryonic hearts cultured as aggregates in suspension. With their culture technique, they obtain two populations of cells which differ in their value of $\dot{V}_{max}$ ("fast" and "slow" cells). The effect of $ScTX_{II}$ depends on the type of cell.

Fast Cells. This population of cells has resting potentials between -40 and -72 mV. $\dot{V}_{max}$ exceeds 15 V/s and is significantly related to the level of the resting potential, reaching a value of 125 V/s at the most negative resting potential. TTX (0.4 μmol/litre) reduces $\dot{V}_{max}$ by about 90%. $ScTX_{II}$ (30 nmol/litre) causes approximately a tenfold increase in the duration of the action potential, while it does not significantly alter $\dot{V}_{max}$. TTX (0.1 μmol/litre) rapidly suppresses the toxin-induced modification of the action potential. D 600, the methoxy derivative of verapamil, at a concentration of 1 μmol/litre, does not prevent prolongation of the action potential by $ScTX_{II}$. These results suggest that $ScTX_{II}$ prolongs the open state of Na channels in cultured cells with expressed Na channels, perhaps by slowing inactivation as in neural Na channels (Sect. 3.6).

Slow Cells. This population of cells has resting potentials between -40 and -62 mV, and $\dot{V}_{max}$ is 10 V/s or less, irrespective of the resting potential, and unaffected by TTX at 0.4 μmol/litre. The low value of $\dot{V}_{max}$, its lack of correlation with the level of the resting potential, and its insensitivity to TTX indicate that the upstroke of the action potential is not mediated by fast Na channels. In these cells, $ScTX_{II}$ (30 nmol/litre), in addition to prolonging the repolarization phase of the action potential, increases $\dot{V}_{max}$ significantly from 8.6 to 36.8 V/s on average, while having

no effect on the level of the resting membrane potential. *Both* effects are suppressed by TTX (0.1 μmol/litre). $ScTX_{II}$ restores action potentials in cells when electrical activity was previously reduced by D 600 (0.5 μmol/litre). *Bernard* and *Couraud* (1979) conclude that $ScTX_{II}$ increases $\dot{V}_{max}$ (in slow cells) and prolongs the repolarization phase of the action potential (in both types of cells) by retarding the inactivation of Na channels. The fact that $ScTX_{II}$ conveys TTX sensitivity to the electrical activity of slow cells shows that these normally TTX-resistant cells contain silent Na channels (Sect. 3.9).

Effect on Na and Ca Influx. $ScTX_{II}$ causes a TTX-sensitive increase of Na influx in embryonic cardiac cells (*Couraud* et al. 1976), as might be expected from the electrophysiological actions described above. The influx of Ca ions is enhanced as well. The effect of Na channel-gate toxins on Ca influx in cultured heart cells is discussed in the section on Na-Ca exchange (Sect. 5.3.2).

4.7 Sea Anemone Toxins

4.7.1 Toxin II from Anemonia sulcata

Isolated Mammalian Heart. In the heart-lung preparation of the cat, ATX_{II} produces a positive inotropic effect (*Alsen* et al. 1976). The threshold concentration is approximately 2 nmol/litre, and a maximum positive inotropic effect occurs at around 100 nmol/litre. In the inotropic concentration range, ATX_{II} has no significant chronotropic action. Toxic symptoms occur at 160 nmol/litre. They are characterized by periods of ventricular fibrillation alternating with periods of normal spontaneous rhythm. In the isolated guinea-pig heart stimulated at 3 Hz, ATX_{II} produces a half-maximal positive inotropic effect at about 5 nmol/litre on the ventricle and atrium (*Alsen* et al. 1976). Arrhythmia occurs at 25 nmol/litre ATX_{II}.

Atrium. In guinea-pig atrium, ATX_{II} (10 nmol/litre) prolongs the repolarization phase of the action potential at all levels of membrane potential and produces a positive klinotropic and positive inotropic effect (*Ravens* 1976). The prolonged phase of repolarization is associated with a prolongation of the relaxation phase of the isometric contraction.

Ventricular Muscle. Ravens (1976) analysed the effects of ATX_{II} on the transmembrane electrical activity and contraction of the isolated guinea-pig papillary muscle contracting at 1 Hz. At concentrations of 5–20 nmol/

litre, the toxin produces graded positive klinotropic and positive inotropic effects. They reach steady-state in about 30 min and disappear within about 1 h of superfusion with toxin-free solution. Inotropic concentrations induce a prolongation of the action potential at negative levels of membrane potential. This prolongation is only partially reversed during a 1-h washout period. Concentrations up to 20 nmol/litre leave the resting membrane potential and the amplitude of the action potential unaffected. $\dot{V}_{max}$ is slightly reduced by 20 nmol/litre ATX_{II}. The prolonged repolarization phase is associated with a prolongation of the relaxation phase of the isometric contraction. The effects of ATX_{II} are not modified by catecholamine depletion with reserpine. TTX (0.5 μmol/litre) completely and reversibly inhibits the effect on action potential duration and the positive inotropic effect. ATX_{II} (10 nmol/litre) fails to prolong the action potential and to increase force of contraction if extracellular K is raised from 2.7 to 14.7 mmol/litre, and raising the K concentration after the effects of ATX_{II} have developed causes their disappearance. The sensitivity to TTX of the toxin-modified repolarization phase suggests that it is due to the persistent activation of Na channels. This might result from a delayed inactivation of the Na channels, which is the main effect of ATX_{II} on neural Na channels (Sect. 3.7.1).

Cardiac Cells in Culture. Romey et al. (1980) analysed the effects of ATX_{II} on cardiac cell aggregates in culture obtained from 3- and 16-day-old chick embryo hearts, respectively. The younger cells exhibit low values of $\dot{V}_{max}$ and their action potential is insensitive to TTX; they will be referred to as slow cells. The older cells show values of $\dot{V}_{max}$ greater than 100 V/s and their action potentials are blocked by TTX (0.1 μmol/litre); these cells will be called fast cells. Very similar to scorpion toxin (Sect. 4.6.2), ATX_{II} produces different effects on slow and fast cells. In fast cells, ATX_{II} only prolongs the falling phase of the action potential and thus produces qualitatively the same effect as observed in adult guinea-pig myocardial cells (*Ravens* 1976). In slow cells, ATX_{II} produces an increase of $\dot{V}_{max}$ (from 12.5 to 25 V/s on average), in addition to prolonging the falling phase of the action potential. The increase of $\dot{V}_{max}$ occurs under conditions where any steady-state inactivation of the Na channels is prevented by a long-lasting hyperpolarizing prepulse. In fast cells, TTX (0.1 μmol/litre) slows $\dot{V}_{max}$ and abolishes the effect of ATX_{II} on the repolarization phase. The same concentration of TTX blocks the two effects of ATX_{II} on the action pontential of slow cells, namely the increase in $\dot{V}_{max}$ and the prolongation of the falling phase. These results as well as those obtained with scorpion toxin (Sect. 4.6.2) suggest that the polypeptide toxins slow Na inactivation in fast cells, thereby prolonging the repolarization phase of the action potential. In slow cells,

the polypeptide toxins cause the appearance of otherwise silent Na channels, as suggested by the TTX-sensitive increase in $\dot{V}_{max}$, and these Na channels seem to inactivate only slowly, as shown by the TTX-sensitive prolongation of the repolarization phase. Thus, ATX_{II} reveals Na channels which are not normally expressed in embryonic heart cells derived at an early stage of development. In addition, *Romey* et al. (1980) showed that ATX_{II} exerts a positive inotropic effect on embryonic cardiac cells along with its effect on the action potential. The inotropic effect is blocked by TTX (0.1 μmol/litre) suggesting that it is causally related to the modification of the Na channels by ATX_{II}. The link may be increased Na-Ca exchange across the sarcolemma, as indicated by the observation that ATX_{II} increases Ca influx as well as Na influx (*Romey* et al. 1980). The effect of Na channel-gate toxins on Ca influx is discussed in the section on Na-Ca exchange (Sect. 5.3.2).

4.7.2 Anthopleurin A

Sinoatrial Node. At concentrations of 5 or 30 nmol/litre, anthopleurin A has no significant effect on the spontaneous heart rate of isolated atria from the rabbit, cat, guinea pig or rat (*Shibata* et al. 1976).

Atrium. Anthopleurin A produces a reversible positive inotropic effect on isolated left atria from the rabbit, cat, guinea pig or rat at a contraction frequency of 1.6 Hz (*Shibata* et al. 1976). Half-maximally effective concentrations are 1–3 nmol/litre. The effect is not modified by propranolol (1 μmol/litre). TTX (2 μmol/litre) decreases the sensitivity of guinea-pig atria to the positive inotropic action of anthopleurin A (*Shibata* et al. 1978). Anthopleurin A (50 nmol/litre) does not alter the cAMP content of guinea-pig atria (*Shibata* et al. 1976).

Purkinje Fibres. Shimizu et al. (1979) studied the effects of anthopleurin A on the transmembrane electrical activity of isolated canine Purkinje fibres and ventricular muscle fibres in a preparation containing fibres of the right bundle branch and of the anterior papillary muscle. In Purkinje fibres stimulated at 1.67 Hz, anthopleurin A (3.9–39 nmol/litre) prolongs the repolarization phase of the action potential by prolonging the duration of the plateau phase. It has little effect on the subsequent phase of repolarization, i.e. the times to 50% and 90% repolarization are prolonged to an equal extent. The refractory period is increased along with the prolongation of the plateau phase of the action potential. At high concentrations of the polypeptide (19–39 nmol/litre), premature action potentials develop from the prolonged repolarization phase at potential levels between −10 and −50 mV. Up to the highest concentration tested

(39 nmol/litre), anthopleurin A does not significantly affect the resting membrane potential, amplitude or $\dot{V}_{max}$ of the action potential. In concentrations below 19 nmol/litre, anthopleurin A produces no significant change in the firing rate of spontaneously active Purkinje fibres. *Shimizu* et al. (1979) did not test whether TTX inhibits the effect of anthopleurin A on the repolarization phase, as has been observed in the presence of batrachotoxin (*Hogan* and *Albuquerque* 1971) or veratrine (*Arbel* et al. 1975). An inhibition by TTX would be expected if anthopleurin A slows the inactivation of Purkinje fibre Na channels as it does that of neural Na channels (Sect. 3.7.2) and ventricular myocardial Na channels.

Ventricular Muscle. Anthopleurin A increases the force of contraction of isolated papillary muscles and ventricular strips of the guinea-pig heart driven at 1.6 Hz (*Shibata* et al. 1976). Half-maximally effective concentrations are 4.4–4.8 nmol/litre. At a threshold concentration near 2 nmol/litre, anthopleurin A also increases the force of the isolated cat papillary muscle stimulated at 1 Hz (*Scriabine* et al. 1979): As shown in this study the positive inotropic and positive klinotropic effects of anthopleurin A are associated with a marked prolongation of the relaxation phase (at 64 nmol/litre). Arrhythmia is produced by 120 nmol/litre. As in dog Purkinje fibres, but to a smaller extent, anthopleurin A increases the action potential duration and the refractory period in dog ventricular muscle fibres (*Shimizu* et al. 1979): The prolongation of the repolarization phase is already manifest at positive levels of membrane potential. In this tissue, anthopleurin A, at concentrations of up to 39 nmol/litre, does not affect the resting potential, $\dot{V}_{max}$, or the amplitude of the action potential. Using a voltage-clamp technique (single sucrose gap) on the isolated guinea-pig papillary muscle, *Hashimoto* et al. (1980) have recently shown that anthopleurin A (9.7–39 nmol/litre) induces a maintained inward current during depolarizing pulses superimposed on the outward current and reaching a maximum value between -40 and -20 mV. The inward current represents a persistent component of Na flux through Na channels since it is blocked by TTX. TTX likewise abolishes the prolongation of the action potential produced by anthopleurin A in guinea-pig papillary muscle (*Hashimoto* et al. 1980). *Ochi* and colleagues (*Hashimoto* et al. 1980) were the first to suggest that anthopleurin A produces its positive inotropic effect by increasing Na influx.

Anthopleurin A (50 nmol/litre) does not affect the activity of Na, K-ATPase isolated from guinea-pig or dog hearts, of phosphodiesterase isolated from guinea-pig hearts, and it does not alter the cAMP content of guinea-pig ventricle (*Shibata* et al. 1976).

4.8 Coral Toxin

The cardiac action of goniopora toxin was examined in isolated left atrial preparations of the rabbit (*Fujiwara* et al. 1979). At 30 nmol/litre, the polypeptide toxin prolongs the action potential at all levels of repolarization, while no significant change is observed either in the resting membrane potential, the overshoot or $\dot{V}_{max}$. The effect on action potential duration persists during superfusion with toxin-free solution for 2 h. TTX (1 μmol/litre) abolishes the effect on the replarization phase. At 3–100 nmol/litre goniopora toxin produces a positive inotropic effect that also persists during washout with toxin-free solution. In line with an irreversible binding reaction, the positive inotropic effect during exposure to a given concentration of the toxin does not reach a steady-state, but progresses and is eventually followed by arrhythmia characterized by coupled extrasystoles. Increasing the concentration of the toxin accelerates the development of the positive inotropic effect. Propranolol, phentolamine or atropine (each at 1 μmol/litre) do not modify the positive inotropic effect. The positive inotropic and arrhythmogenic effect are both abolished by TTX (1 μmol/litre). In atria depolarized by an elevated extracellular K concentration (27 mmol/litre) and treated with 0.5 μmol/litre isoprenaline to restore excitability (0.25 Hz), goniopora toxin fails to increase force of contraction. The results suggest that goniopora toxin modifies the cardiac action potential by prolonging the open state of Na channels and that the positive inotropic effect is linked to the increase in sarcolemmal P_{Na}.

5 Sarcolemmal Na Permeability and Myocardial Force of Contraction: Na-Ca Exchange

5.1 Introduction

For an understanding of the positive inotropic action of the Na channel-gate toxins it is necessary to consider the role played normally by Na channels in the excitation-contraction cycle of the heart. The most important function of the Na channels is to allow a rapid conduction of the excitation wave, originating in the sinoatrial or atrioventricular node, to all cells of the atrial and ventricular myocardium. The nearly simultaneous excitation of all atrial and ventricular cells assures the coordinated contraction of these cells, i.e. the function of the heart as a pump. The process which couples sarcolemmal excitation to contraction of the myocardial cell does not seem to depend so much on the activation of Na

channels, but seems to be intimately related to events occurring during the plateau phase of the action potential, notably the Ca inward current (*Beeler* and *Reuter* 1970a,b,c; *New* and *Trautwein* 1972; *Trautwein* et al. 1975) and electrogenic Na-Ca exchange. Thus, contractions can still be elicited if Na channels are blocked by TTX or completely inactivated by sustained depolarization of the sarcolemma. It is nevertheless very likely that Na channels, in addition to permitting the rapid conduction of the cardiac action potential, influence excitation-contraction coupling, because their activation leads to an influx of Na ions into the cell. Several lines of evidence, reviewed here, suggest that an increased concentration of intracellular Na ions promotes an influx of Ca ions across the sarcolemma (Na-Ca exchange). This is expected to result in a positive inotropic effect. Na influx through Na channels is greatly enhanced by the Na channel-gate toxins, because they drastically prolong the time during which the Na channels are in the open state.

The intracellular Na concentration may also affect the Ca transport by organelles of the myocardial cell. If exposed to Na ions, cardiac mitochondria (*Carafoli* and *Crompton* 1978) release Ca. It seems difficult, however, to explain a maintained positive inotropic effect by a mechanism that depletes cellular stores and thus, eventually, the cell of Ca ions. A decrease of the [K]/[Na] ratio may impair Ca uptake by cardiac sarcoplasmic reticulum (*Katz* and *Repke* 1967).

Ravens (1976) suggested an explanation for the positive inotropic effect of Na channel-gate toxins that does not involve changes of intracellular Na concentration. According to this hypothesis, the toxin interacts with a sarcolemmal receptor controlling Na channel gating as well as a plasmalemmal Ca store that releases Ca during contraction. However, since TTX, a selective blocker of Na channels, abolishes the postive inotropic effect of Na channel-gate toxins (see Chap. 4), it is not necessary to postulate that the positive inotropic effect of these toxins results from a modification of structures other than those regulating Na channel gating.

5.2 Na-Ca Exchange in the Squid Giant Axon

The giant axons of certain species of squid are particularly suitable for studying the role of intracellular Na in regulating Ca flux across an excitable membrane, because $[Na]_i$ can be selectively altered experimentally either by injection of a Na salt into the axoplasm of an intact axon (*Baker* et al. 1969) or by changing the concentration of Na in the solution used to perfuse an axon intracellularly (*Di Polo* 1979). *Baker* et al. (1969) showed that the intraaxonal injection of NaCl to raise $[Na]_i$ by 67 or 120 mmol/litre in axons of *Loligo forbesi* previously depleted of

intracellular Na causes an increase in the unidirectional influx of Ca ions by factors of 2 and 4, respectively. These experiments were performed with an external solution in which most of the Na was replaced by Li ions (Li-seawater). In other experiments of their study $[Na]_i$ was raised by prolonged stimulation at high frequency. This resulted in an increase of Ca influx irrespective of whether the influx was measured in Na-, Li- or dextrose-seawater. The additional demonstration of a component of Na efflux, which is insensitive to cardioactive steroids, but activated by external Ca ions, led to the hypothesis that the enhancement of Ca influx by internal Na involves a transmembrane ion exchange in which the exit of Na ions is linked to the entry of Ca ions (*Baker* et al. 1969).

A detailed analysis of the component of Ca influx enhanced by intracellular Na ions was performed by *Di Polo* (1979) using the technique of intracellular dialysis on axons of the tropical squid *Dorytheutis plei*. The main findings of this study may be summarized as follows: An increase of $[Na]_i$ of 0–120 mmol/litre increases Ca influx, and the Na_i-dependent Ca influx requires a threshold level of $[Ca^{2+}]_i$ of 40 nmol/litre and increases as $[Ca^{2+}]_i$ is raised to 0.8 μmol/litre. The Na_i-dependent Ca influx requires the presence of ATP. The results of *Baker* et al. (1969) indicate that the entry of one Ca ion may be linked to the exit of more than two Na ions, which would make Na-Ca exchange sensitive to the membrane potential. In line with this prediction, both the Na efflux component (*Baker* and *McNaughton* 1976) and the Ca influx component (*Di Polo* 1979) constituting the Na-Ca exchange under physiological conditions, i.e. in the presence of ATP, were found to be augmented by depolarization.

Baker et al. (1969) pointed out that the exchange of external Ca for internal Na which they observed may represent ion movements in a direction opposite to that occurring under normal conditions. The normal function of a Na-Ca exchange system might be to pump Ca out of the cell in exchange for external Na, as suggested by *Reuter* and *Seitz* (1968). In this case the energy for the uphill transport of Ca ions from the cell could be provided by the downhill movement of Na ions, and the transmembrane gradient for Na ions would be maintained by the Na pump. Interestingly, although a Ca efflux-Na influx system operates in axons poisoned with cyanide (*Blaustein* and *Hodgkin* 1969), later work has shown that 50%–90% of the Ca efflux from unpoisoned squid axons is independent of external Na ions and requires the presence of ATP (*Baker* and *McNaughton* 1978; *Di Polo* and *Beaugé* 1979).

The effect of intracellular Na ions on unidirectional Ca *efflux* has also been examined. Raising $[Na]_i$ from 5 to 75 mmol/litre in internally dialyzed axons of *Loligo pealei* inhibits the $[Na]_o$-dependent component of Ca efflux (*Blaustein* 1977), but the uncoupled Ca efflux, which may

constitute the main part of Ca efflux from intact axons (*Baker* and *McNaughton* 1978), is insenstitive to changes in intracellular Na concentration (*Di Polo* and *Beaugé* 1979). Thus, an increase of $[Na]_i$ will affect the intracellular Ca concentration of squid axon through an increase of Ca influx with little effect on Ca efflux.

5.3 Na_i-Ca_o Exchange in Cardiac Preparations

5.3.1 Guinea-Pig Atrium

Glitsch et al. (1970) examined the relationship between the intracellular Na concentration and Ca influx in resting left atria of guinea pigs. They produced different levels of $[Na]_i$ by equilibrating the resting muscles in K-rich solution ($[Na]_i$ = 12.5 mmol/kg fibre water, as determined by flame photometry), in normal solution ($[Na]_i$ = 20 mmol/kg) or in cold K-poor, Ca-poor solution ($[Na]_i$ = 60 mmol/kg). An intermediate level of Na_i was established by high-frequency stimulation in K-poor, Ca-poor solution ($[Na]_i$ = 40.5 mmol/kg). Subsequently all preparations were placed for 10 min into a medium containing ^{45}Ca, 0.65 mmol/litre $[K]_o$ to prevent a decrease of $[Na]_i$, and 0.7–7 μmol/litre acetylcholine to prevent spontaneous activity of the atria. After the uptake period the preparations were washed for 25 min with inactive solution before the radioactive analysis was performed. Under these conditions, the increase of $[Na]_i$ was associated with an increase of Ca uptake by the atria. A nearly fivefold increase in $[Na]_i$ caused in increase in Ca uptake by a factor of about 2.6.

5.3.2 Cardiac Cells in Culture

To define the relationship between Na fluxes and Ca fluxes in myocardial cells an approach different from that of *Glitsch* et al. (1970) was used by *Fosset* et al. (1977). They analysed the Ca influx induced by veratridine in monolayer cultures of cardiac myoblasts obtained from 10-day-old chick embryos. Although these authors did not report the actual changes of $[Na]_i$ in their experiments, the various concentrations of veratridine which they applied in a medium containing Na ions (140 mmol/litre) and ouabain increased Na influx and presumably caused a graded increase of $[Na]_i$. No information on the transmembrane potential of the myoblasts is given in this article, but the work by *Sperelakis* and *Lehmkuhl* (1968) and *Sperelakis* and *Pappano* (1969) suggests that either ouabain or the lowest effective concentration of veratridine (1.5 μmol/litre) used by *Fosset* et al. (1977) caused permanent depolarization and inexcitability and thus eliminated possible complications resulting from spontaneous

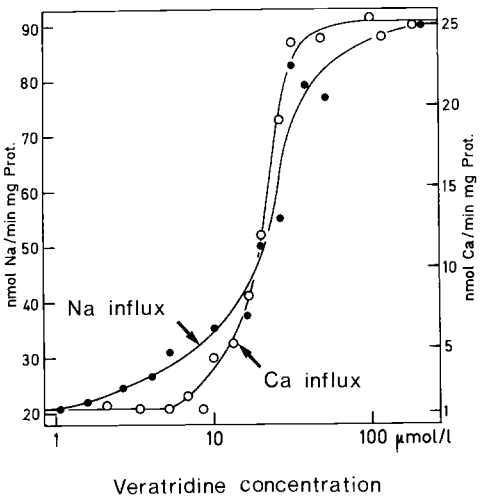


Fig. 12. Concentration-effect relationships for the enhancement of Na influx and Ca influx by veratridine in chick embryonic cardiac cells in culture. The experiments were performed in the presence of 0.5 mmol/litre ouabain. (Drawn after *Fosset* et al. 1977)

activity of cultured heart cells. *Fosset* et al. (1977) showed that veratridine increases Ca influx up to 25-fold (Fig. 12). They could exclude the possibility that this influx uses Na channels opened by veratridine, because veratridine fails to increase Ca influx if external Na is replaced by Li ions, i.e. under conditions where veratridine retains the ability to open Na channels thereby allowing the influx of Li ions (*Sperelakis* and *Pappano* 1969). However, because it is abolished by TTX, the veratridine effect on Ca influx does require the opening of Na channels. Veratridine-induced Ca influx is insensitive to manganese or cobalt ions or to the compound D 600 at concentrations at which these substances normally inhibit slow Ca channels. Thus, the veratridine-induced Ca influx is coupled to the presence of an inwardly directed Na gradient and open Na channels, but the Ca ions enter the cell neither through Na channels nor through slow Ca channels. These findings strongly suggest the existence in cardiac cells of a Ca uptake mechanism that is increased by intracellular Na accumulation (*Fosset* et al. 1977).

Results very similar to those of *Fosset* et al. (1977) were obtained by *Couraud* et al. (1976) who used $ScTX_{II}$. The latter authors suggest that the sarcolemma of cardiac cells in culture contains two types of Ca channels, the well-known slow Ca channel and a Ca channel that is "coupled to the passive Na action potential ionophore" and therefore affected by the toxin. This interpretation disregards the observation by the authors themselves that ouabain greatly enhances the effect of $ScTX_{II}$ on Ca influx, an observation which points to the importance of intracellular Na accumulation in linking the effect of the scorpion toxin on Na channels to its effect on Ca influx. It appears that all the results of *Couraud* et al. (1976) are compatible with the concept that $ScTX_{II}$ increases Na influx

by opening Na channels and thereby indirectly activates an exchange of intracellular Na for extracellular Ca ions. An increase of both Na and Ca influx is also produced by ATX_{II} (*Romey* et al. 1980).

5.3.3 Purkinje Fibres

The experiments of *Glitsch* et al. (1970), *Couraud* et al. (1976) and *Fosset* et al. (1977) do not clarify whether the activating effect of intracellular Na ions on Ca influx involves an actual exchange of internal Na ions for external Ca ions, as in the squid giant axon (*Baker* et al. 1969). If the cardiac sarcolemma possesses a Na-Ca exchange system, raising the extracellular Ca concentration will increase Na efflux thereby reducing the intracellular Na concentration under conditions where $[Na]_i$ is not controlled by the Na pump. Using Na-sensitive glass micro-electrodes, *Deitmer* and *Ellis* (1978) indeed showed that raising the bath concentration of Ca produces a decrease in the intracellular Na activity of sheep heart Purkinje fibres even when the Na-K pump is inhibited by strophantidin.

5.3.4 Myocardial Contraction in Low Extracellular Na

If extracellular Na is reduced in the presence of extracellular Ca, a contracture develops which is associated with a large increase in the influx of Ca into the heart cells (*Niedergerke* 1963). A study on frog atrial trabeculae revealed that this contracture is transient and, after spontaneous relaxation, even a 100-fold increase in the bathing Ca concentration (from 0.1 to 10 mmol/litre) fails to induce any increase in tension (*Chapman* 1974). The results obtained by *Chapman* (1974, 1979) stongly suggest that the increase in Ca influx resulting from the reduction of $[Na]_o$ critically depends on the presence of Na inside the cells. This points to the existence of a Na-Ca exchange mechanism.

Voltage-clamp experiments on frog atrial trabeculae (*Benninger* et al. 1976; *Horackova* and *Vassort* 1979a) indicate that the tonic component of tension (Sect. 6.1) results from a transmembrane Na-Ca exchange that is voltage-dependent and therefore activated by the depolarization used to elicit tonic tension. The tonic component of tension exists in addition to a phasic component that is related to both I_{si} and intracellular Ca stores (*Horackova* and *Vassort* 1976, 1979b).

5.3.5 Inotropic Effect of Cardioactive Steroids

If one accepts the view that cardioactive steroids produce their positive inotropic effect by inhibiting the sarcolemmal Na pump, one has to conclude that the myocardial cell possesses a mechanism that translates some

consequence of this membrane effect into an enhancement of the intracellular Ca transient during contraction (*Allen* and *Blinks* 1978) and thus into a positive inotropic effect. One consequence of the Na pump inhibition by cardioactive steroids is an increase in intracellular Na concentration (*Lee* et al. 1980). A Na-Ca exchange in the sarcolemma would thus provide exactly the mechanism to couple an inhibition of the Na pump to a positive inotropic effect. *Akera* and *Brody* (1978) and *Reiter* (to be published) reviewed the evidence in support of the theory that the positive inotropic effect of cardioactive steroids results from their effect on $[Na]_i$ secondary to the inhibition of the Na pump.

5.3.6 Cardiac Membrane Vesicles

A transmembrane Na-Ca ion exchange was demonstrated in membrane vesicles of presumed sarcolemmal origin. The vesicles were obtained by centrifugation techniques from the ventricle of the heart of the rabbit (*Reeves* and *Sutko* 1979), dog (*Pitts* 1979), and ox (*Miyamoto* and *Racker* 1980). Recent studies indicate that this Na-Ca exchange involves the exchange of more than two Na ions with one Ca ion and is thus electrogenic (*Reeves* and *Sutko* 1980; *Philipson* and *Nishimoto* 1980). Na channel-gate toxins have apparently not been used to alter Na fluxes of cardiac membrane vesicles. The demonstration of a toxin-activated and TTX-inhibited Na flux would provide strong evidence for the sarcolemmal nature of such vesicles. The need for further purification of cardiac sarcolemmal membranes has been stressed by *Sulakhe* and *St. Louis* (1980).

6 Comparison of Cardiac Actions of Na Channel-Gate Toxins with Those of Other Positive Inotropic Drugs

As shown in Chap. 4, the cardiac action of Na channel-gate toxins has been analysed and characterized in terms of their influence on mechanical and electrical activity and on transsarcolemmal ion movements. The purpose of the following paragraphs is to compare some of these effects with those of other classes of positive inotropic agents on the same aspects of myocardial function. This will serve to reinforce the conclusion that Na channel-gate toxins represent a separate class of positive inotropic substances characterized by a distinct mode of action. The discussion deals mainly with effects observed in isolated ventricular preparations of the mammalian heart.

6.1 Isometric Contraction Curve

Na channel-gate toxins increase the force of myocardial contraction by accelerating the rate of force development, i.e. by a positive klinotropic effect (Figs. 2, 3). Hence, they act by increasing the intensity of the "active state" of the contractile elements and differ in this respect from the relatively few interventions which, like the addition of fluoride, produce a positive inotropic effect by prolongation of the active state (*Reiter* 1972a).

Na channel-gate toxins produce variable effects on the relaxation phase of the isometric contraction. The relaxation time may be prolonged to such an extent that total contraction time is lengthened (Fig. 2, veratridine), or the prolongation may just compensate a simultaneous abbreviation of time to peak force resulting in a nearly unchanged total contraction time (Fig. 3, germitrine). The very marked prolongation of relaxation time, causing an increase in total contraction time, is observed in the presence of those toxins which slow repolarization during, or shortly after, the plateau phase of the action potential (e.g., cevadine, veratridine, batrachotoxin, ATX_{II}, anthopleurin A, goniopora toxin; see Chap. 4 for references). In contrast, relatively little effect on the total contraction time is produced by those toxins inducing a slight depolarization of the resting membrane potential (e.g., germitrine, grayanotoxins). It is well known that an electrical prolongation of the repolarization phase (by injecting a depolarizing current with the sucrose-gap technique), at a level positive to about -30 mV, is associated with prolonged relaxation phase of the contraction (*Morad* and *Trautwein* 1968). The resulting component of mechanical activity is maintained as long as the membrane is kept at the depolarized level of membrane potential and is therefore called "tonic" tension (*Morad* and *Goldman* 1973). The markedly prolonged relaxation phase observed with some of the Na channel-gate toxins can thus be explained in terms of the known dependence of mechanical activity on membrane potential. Recent studies indicate that Na channel-gate toxins may prolong relaxation time by an additional mechanism related to the increase in intracellular Na concentration rather than to the prolonged repolarization phase. *Vassort* and colleagues (*Roulet* et al. 1979) have shown that veratrine prolongs the relaxation time of frog atrial trabeculae even if the preparation is activated by voltage-clamp depolarizations of constant duration, i.e. if the prolongation of the action potential produced normally by veratrine is prevented. According to *Roulet* et al. (1979), the relaxation phase is directly influenced by an electrogenic sarcolemmal Na-Ca exchange mechanism. An increase in Na_i is thus expected to decrease the efficiency of the Na-Ca exchange to restore the low diastolic level of the free intracellular Ca concentration

after repolarization. This interpretation could also explain the prolongation of relaxation time produced by Na channel-gate toxins which, like germitrine (Fig. 3), prolong the action potential only at membrane potential levels far negative to −30 mV (Sect. 4.2.5).

Na channel-gate toxins have never been observed to abbreviate total contraction time by shortening relaxation time. Such a relaxant effect is typically produced by inotropic agents which elevate the cellular concentration of cAMP, for instance by β-adrenoceptor agonists, histamine, or phosphodiesterase inhibitors (*Korth* 1978; *Scholz* 1980). While the inotropic effect of the toxins clearly differs in this respect from the cAMP-increasing drugs, it is difficult, if not impossible, to distinguish certain other positive inotropic agents from Na channel-gate toxins solely on the basis of the modification of the isometric contraction curve. Thus, like germitrine, the cardioactive steroid dihydro-ouabain produces a positive klinotropic and inotropic effect with little effect on total contraction time (*Reiter* 1972b).

6.2 Blockade of Sarcolemmal Na Channels by Tetrodotoxin

In the absence of a competitive antagonist for Na channel-gate toxins which lacks intrinsic activity (cf. Sect. 3.10.2), the most specific way to test whether a positive inotropic agent acts by prolonging the open state of Na channels is to use the non-competitive antagonist TTX (or STX). TTX occludes Na channels and thereby prevents the effects of Na channel-gate toxins on membrane potential and Na and Ca influx. In guinea-pig papillary muscle, a TTX concentration which reduces $\dot{V}_{max}$ by 50% (10 μmol/litre) has no inhibitory influence on the positive inotropic effect of noradrenaline or theophylline and slightly inhibits that of dihydro-ouabain, but prevents the positive inotropic effect of half-maximally effective concentrations of cevadine, veratridine, and germitrine (Fig. 10). The fact that TTX fails to affect the positive inotropic effect of noradrenaline and theophylline is in agreement with the view that these agents act by increasing the cellular concentration of cAMP and that such an increase mediates the enhancement of slow inward Ca current and of the amount of Ca releasable from, and sequestered by an intracellular Ca store (for references see *Scholz* 1980). TTX lacks an effect on slow inward Ca current and is not expected to gain access to the intracellular space during extracellular application (Sect. 4.2.5). Furthermore, even if applied directly to the sarcoplasmic reticulum as in the experiments on skinned cardiac fibres by *Fabiato* and *Fabiato* (1973), TTX does not affect the Ca release-sequestration cycle as judged from the unchanged mechanical activity. The finding that TTX less effectively

inhibits the positive inotropic effect of dihydro-ouabain than that of ceveratrum alkaloids, although the action of both cardioactive steroids and Na channel-gate toxins depends on Na influx, has been discussed in Sect. 4.2.5.

The use of TTX for the classification of inotropic mechanisms requires some precautions. It is necessary to rule out that the agent in question acts primarily on neural Na channels to cause the release of noradrenaline from intracardiac nerve endings. Such an effect is also blocked by TTX; in fact by concentrations much lower than those required to block sarcolemmal Na channels. Among the substances covered in this review only scorpion toxins were found to act mainly by a presynaptic mechanism. In the case of veratridine and cevadine, a neural effect is also demonstrable, but requires concentrations in excess of those affecting sarcolemmal Na channels (Sect. 4.2.5). It is also important to note that the pattern of inhibition by TTX depends on whether the Na channel-gate toxin acts reversibly or irreversibly. In the case of reversibly acting toxins, like veratridine (*Honerjäger* and *Reiter* 1975) or ATX_{II} (*Ravens* 1976), TTX produces a stable inhibition if applied after the effects of the gate toxins have attained steady-state. With irreversibly acting toxins, like batrachotoxin (*Honerjäger* and *Reiter* 1977b), the effect of TTX, when applied in a submaximally effective concentration, is to delay the development, but not to prevent the effect of the Na channel-gate toxin. It appears that the change in cardiac membrane potential, such as the delay of the repolarization phase, requires only a small fraction of all Na channels to be modified (*Horackova* and *Vassort* 1973; *Trautwein* 1973). Thus TTX concentrations of 50 μmol/litre or less, which reduce $\dot{V}_{max}$ only up to 80% in guinea-pig papillary muscle (*Baer* et al. 1976), leave a significant fraction of Na channels unblocked, and this unblocked fraction of Na channels may suffice to mediate a prolongation of the action potential or decrease of the resting potential in the presence of a very high concentration of Na channel-gate toxin (*Honerjäger* and *Reiter* 1977b). *Ito* et al. (1979) observed that 1 μmol/litre TTX failed to restore the membrane potential in guinea-pig papillary muscle depolarized by the irreversibly acting palytoxin, and they conclude that "the partial antagonism by TTX implies the possibility that PTX [palytoxin] did not act 'specifically' on the Na channel and produced a leaky state". However, their findings that TTX delayed the onset of the palytoxin-induced depolarization and that reduction of $[Na]_o$ to 12.3 mmol/litre did restore the membrane potential in the presence of palytoxin are compatible with the view that palytoxin acts entirely by selectively opening Na channels.

6.3 Contraction Frequency

The frequency dependence of the positive inotropic effect of veratridine (*Honerjäger* and *Reiter* 1975) or germitrine (*Honerjäger* and *Reiter* 1977a) differs from that of cAMP-increasing agents and, in certain details, from that of the cardioactive steroid dihydro-ouabain. The ceveratrum alkaloids lack a positive inotropic effect on the rested-state contraction (Sect. 4.2.5) whereas threshold effective concentrations at 1 Hz also increase the peak force of the rested-state contraction in the case of adrenaline and theophylline (cat papillary muscle; *Bereşewicz* and *Reuter* 1977) and noradrenaline and N^6-2′-O-dibutyryl cAMP (guinea-pig papillary muscle; *Seibel* et al. 1978). Force-frequency relationships obtained with concentrations of dihydro-ouabain that are submaximally effective at 1 Hz (*Ebner* and *Reiter* 1977) are virtually identical to those obtained with veratridine or germitrine. Like the ceveratrum alkaloids, these concentrations of dihydro-ouabain lack an effect on the rested-state contraction and produce a progressively increasing positive inotropic effect as the frequency is raised stepwise from 0.003 Hz to 0.5 Hz. Ceveratrum alkaloids lack a positive inotropic effect on the rested-state contraction even at concentrations exceeding the maximally effective concentration at a Hz by more than one order of magnitude (Sect. 4.2.5). In the cases of veratridine or cevadine, the demonstration of this lack of positive inotropic effect requires the elimination of their presynaptic effect (Sect. 4.2.5). In contrast, dihydro-ouabain increases the force of the rested-state contraction in concentrations that are maximally or supramaximally effective at 1 Hz (*Ebner* and *Reiter* 1977). In the cases of veratridine and germitrine, the phase of increased P_{Na} is linked to the action potential, which explains the absence of a positive inotropic influence under resting conditions. However, it is conceivable that agents which increase resting P_{Na}, like grayanotoxin I in rabbit atrial fibres (*Seyama* 1978), also increase the rested-state contraction.

6.4 Reduction of Extracellular Na Concentration

Reducing the extracellular Na concentration by one-half greatly diminishes the positive inotropic effect of cardioactive steroids, whereas the inotropic effect of catecholamines is unaffected (*Reiter* 1972a). Using this test, the ceveratrum alkaloids veratridine and cevadine were shown to be influenced in their effect on guinea-pig papillary muscle like the steroids (*Reiter* 1963), pointing to the importance of Na influx for the action of the alkaloids.

6.5 Variation of Extracellular K Concentration

The level of the extracellular K concentration may influence the positive inotropic effect of Na channel-gate toxins by at least two different mechanisms, through its effect on transmembrane potential and on the activity of the Na-K pump. *Ravens* (1976) showed that ATX_{II} failed to prolong the action potential and to produce a positive inotropic effect in guinea-pig papillary muscle if the K concentration was raised from 2.7 to 14.7 mmol/litre. The elevated K concentration caused a fall of the resting potential to about −50 mV, which is expected to inactivate normal Na channels (*Beeler* and *Reuter* 1970a). In contrast to ATX_{II}, agents which increase the cellular cAMP concentration retain their positive inotropic effect and increase amplitude and duration of the action potential through their effect on I_{si} if Na channels are inactivated by depolarization (for references see *Scholz* 1980). Like the positive inotropic effect of ATX_{II}, that of goniopora toxin on rabbit atrial preparations is prevented at an elevated (27 mmol/litre) extracellular K concentration (*Fujiwara* et al. 1979).

Reducing the K concentration below 5.9 mmol/litre enhances the positive inotropic effect of veratridine (*Honerjäger* and *Reiter* 1975). The associated hyperpolarization of the resting potential from about −80 mV at 5.9 mmol/litre $[K]_o$ has presumably little effect on the level of steady-state inactivation of the Na channels, since this seems to be already minimal at −80 mV (*Chen* et al. 1975). It seems more likely that the synergistic action of K withdrawal and veratridine is related to an inhibition of the Na pump by reduction of $[K]_o$, which is expected to increase the extent of the veratridine-induced intracellular Na accumulation and therefore the positive inotropic effect. This test does not discrimiate between veratridine and dihydro-ouabain, the positive inotropic effect of which is likewise enhanced by a reduction in extracellular K concentration (*Reiter* et al. 1966). In contrast, the inotropic effect of adrenaline (*Reiter* et al. 1966) or theophylline (*Scholz* and *de Yazikof* 1971) is not increased by a lowering of extracellular K concentration.

6.6 Inhibition of the Na Pump by Dihydro-Ouabain

Dihydro-ouabain potentiates the positive inotropic effect of veratridine (*Honerjäger* and *Reiter* 1975) and germitrine (*Honerjäger* and *Reiter* 1977a), whereas veratridine and noradrenaline act additively. The increment in intracellular Na concentration induced by Na channel-gate toxins is normally counteracted by the Na-K pump, the activity of which is not directly influenced by Na channel-gate toxins. Hence, an inhibition of

the Na pump will enhance the extent of the increment in intracellular Na concentration caused by agents which increase Na influx. The synergistic action of ceveratrum alkaloids and a specific Na pump inhibitor thus indirectly points to the critical role of intracellular Na concentration for the positive inotropic effect of Na channel-gate toxins.

6.7 Propranolol

(±) Propranolol (5 μmol/litre) shifts the concentration-effect curve for the positive inotropic effect of veratridine to the right by a factor of 2 (*Honerjäger* and *Reiter* 1975). The same concentration of (+) propranolol causes the same degree of inhibition, although this isomer has only about 1/60 the activity of racemic propranolol in blocking the effects of β-adrenoceptor activation (*Howe* and *Shanks* 1966). The antagonism is thus unrelated to the blockade of β-adrenoceptors by propranolol, but is readily explained by the inhibitory effect of propranolol on myocardial Na channels which is identical for both isomers of propranolol (*Pollen* et al. 1969). Similarly, the antagonism of the positive inotropic effect of grayanotoxins by propranolol (10 μmol/litre, Sect. 4.5.2) may be entirely due to the blockade of Na channels by propranolol.

7 Summary and Conclusions

In recent years the detailed analysis of the mode of action of various Na channel-gate toxins has revealed that they prolong the open state of Na channels by mechanisms that differ in some detail. The alkaloids veratridine, batrachotoxin, and aconitine modify both activation and inactivation characteristics, while certain polypeptide toxins like ATX_{II} predominantly affect Na inactivation and one of the scorpion venoms selectively modifies Na activation. These mechanisms were clarified by voltage-clamp experiments on single nerve fibres. Less direct experiments were performed on myocardial cells, but they all indicate that the Na channels of cardiac sarcolemma are also modified by various Na channel-gate toxins. The modification of cardiac Na channels results in a prolongation of the repolarization phase of the cardiac action potential which, depending on the particular toxin, may become evident at any potential level between the plateau and close to the resting potential. In embryonic cardiac cells, the application of Na channel-gate toxins revealed the presence of normally silent TTX-sensitive Na channels.

The prolonged phase of repolarization provides the depolarizing stimulus for coupled premature action potentials and thus explains the arrhythmogenic effect of Na channel-gate toxins. This type of arrhythmia is not specific for this kind of substances, but it is the consequence of a selective alteration of Na channel kinetics and therefore is completely inhibited by TTX.

With the exception of aconitine, all Na channel-gate toxins with a demonstrable effect on myocardial Na channels were also shown to produce a positive inotropic effect in the prearrhythmic stage of action. Thus, a direct positive inotropic effect is observed in the presence of steroid alkaloids, diterpenoids, and polypeptides. Such a uniform response of the contractile mechanisms in the myocardial cell to substances of widely differing chemical structure suggests that the inotropic effect is somehow related to the known common mechanism of action of these toxins, i.e. the modification of the gating system of sarcolemmal Na channels.

The experimental evidence surveyed indicates that the mechanism of the positive inotropic effect of Na channel-gate toxins involves the following chain of subcellular events: The initial step is the alteration of the sarcolemmal Na channels by the toxin. The essentiality of this step is deduced from the observation that TTX prevents or abolishes the positive inotropic effect at concentrations which also prevent or abolish the electrophysiologic changes resulting from the Na channel modification. The selectivity of action of TTX in blocking Na channels is well established. In addition, TTX was shown to cause comparatively little or no antagonism to the positive inotropic effect of drugs that do not affect Na channel kinetics (dihydro-ouabain, noradrenaline, theophylline).

A cause-effect relationship requires that the causative event precedes the effect. This is clearly shown for Na channel-gate toxins in that the modified repolarization phase precedes the positive inotropic effect.

The slowing of Na channel kinetics prolongs the time during which Na ions flow into the cell. In addition, the prolonged repolarization phase indirectly affects voltage- and time-dependent ionic currents other than Na current. The change in membrane potential, however, is not essential for the positive inotropic effect. As shown in voltage-clamp experiments on frog atrial trabeculae, the positive inotropic effect is still present if the prolongation of the action potential is prevented by submitting the preparation to depolarizations of constant amplitude and duration. Hence, the inotropic effect is coupled to the increase in Na current induced by the toxins. The importance of Na influx is also indicated by the fact that the positive inotropic effect of Na channel-gate toxins is potentiated by dihydro-ouabain, an inhibitor of Na efflux.

How does an increase in Na influx mediate a positive inotropic effect? A well-documented mechanism is the Na-Ca exchange, which provides an increase in transsarcolemmal Ca influx in response to an elevation of the intracellular Na concentration. In fact, Na channel-gate toxins were used to demonstrate the existence, in cardiac cells, of a Na-Ca exchange mechanism because they are convenient chemical tools to selectively produce an increase of the intracellular Na concentration in small cells.

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Factors Influencing Renal Sodium Reabsorption in Volume Expansion

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1 Introduction

This review discusses factors which influence renal sodium reabsorption in volume expansion. The factors are considered under seven headings: hemodynamic factors; glomerular filtration rate; physical factors; segmental sodium reabsorption; hormonal factors; response of the perinatal kidney to volume expansion; and gastrointestinal or hepatoportal control of renal sodium excretion. The results discussed consist primarily of clearance, micropuncture, microperfusion, and histologic studies. It seems justified to inject a word of caution in regard to interpretation of the data contained herein. In the majority of the studies, the animals were anesthetized, subjected to varying degrees of surgical trauma, and subsequently given an extracellular volume expansion (ECVE). Inasmuch as these maneuvers are not physiologic, extrapolation of the results to normal renal function should be done with caution.

2 Renal Sodium Reabsorption

2.1 Hemodynamic Factors

2.1.1 Renal Blood Flow and Renal Vascular Resistance

Expansion of the extracellular fluid volume (ECFV) by an infusion of isotonic saline has been shown to decrease renal vascular resistance and increase renal blood flow (*Barger* et al. 1961; *Earley* and *Friedler* 1965a; *Wallin* et al. 1971). In initial studies, the extent to which this increased renal blood flow alone results in increased sodium excretion was evaluated in clearance experiments in dogs by infusing vasodilators alone, or in the presence of a saline load (*Earley* and *Friedler* 1965b). The results of these studies demonstrated that in hydropenic animals, increasing renal blood flow by vasodilators alone was accompanied by an increased sodium excretion in spite of an unchanged filtered load. In animals receiving a saline load, increased renal blood flow of the same magnitude was associated with a threefold greater natriuresis. Further studies demonstrated that infusion of iso-oncotic Ringer's solution, designed not to alter plasma protein concentration, resulted in large increases in arterial pressure, renal blood flow, and urinary sodium excretion. If the kidneys were vasodilated with acetylcholine prior to the iso-oncotic Ringer's loading, the additional natriuresis seen with the vasodilated kidney was significantly reduced (*Martino* and *Earley* 1967). These studies were taken as evidence that increases in renal blood flow, per se, can increase

sodium excretion, but can only partially account for the natriuresis accompanying saline loading. The possibility exists, however, that the natriuretic effect seen with vasodilators is due to a direct action of the drug on the renal tubule and that the increased renal blood flow per se is not necessarily natriuretic. For example, secretin is a vasodilator which is not natriuretic (*Marchand* et al. 1977a). Further, small infusions of saline which increase urinary sodium excretion may not result in increases in renal blood flow (*Martino* and *Earley* 1967).

2.1.2 Intrarenal Distribution of Blood Flow

Volume expansion may be associated with increased medullary renal blood flow, since increased sodium excretion is accompanied by a decrease in urinary osmolality and the extraction ratio of *p*-aminohippurate (PAH) (*Earley* and *Friedler* 1964, 1965a; *Friedler* and *Earley* 1966). This decrease in urinary osmolality, which could not be accounted for by changes in either antidiuretic hormone (ADH) or solute excretion, and the assumption that PAH is extracted only from blood perfusing the cortex, led *Earley* and *Friedler* to hypothesize that saline loading in the dog results in an increased medullary blood flow, and that there may be a causal relationship between this intrarenal distribution of blood flow and the associated natriuresis of ECVE. The issue of whether a change in the intrarenal distribution of blood flow is associated with the renal vasodilation seen with volume expansion has subsequently been studied with a variety of techniques. The different techniques used to measure intrarenal blood flow distribution include the use of extractable substances such as PAH, dye dilution, washout of inert gases like ^{85}Kr and ^{133}Xe, H_2 clearance, glomerular basement-membrane antibody technique, and radioactive-labeled microspheres. A description of the different methods and a discussion of their validity is given by *Barger* and *Herd* (1973). Initial studies which utilized the inert-gas washout or H_2 clearance method to evaluate intrarenal distribution of blood flow reported that in volume expanded dogs, blood flow in the renal cortex increases, but there was not a significant change in the distribution of blood flow within the cortex (*Munck* et al. 1970; *Loyning* 1974). This finding was essentially confirmed in a subsequent study also carried out in dogs. *Bovee* and *Webster,* utilizing the inert-gas washout method and PAH extraction, reported that the increase in renal blood flow during moderate saline loading is limited to the cortex, but during large saline loads, cortical and noncortical flows increase proportionately (*Bovee* and *Webster* 1971). These findings are in agreement with recent observations of *Velasquez,* who applied the dye-dilution technique for quantitation of intrarenal blood flow distribution, and reported that during the early phase of saline

diuresis renal vasodilatation is confined to the cortex, but during later stages of saline loading noncortical blood flow increases also (*Velasquez* et al. 1973, 1975).

Interestingly, a study in the rat utilizing the X^e washout method found no significant change in either total renal blood flow or outer cortical blood flow after volume expansion with saline (*Kinney* and *DiScala* 1974). More recent results evaluating intrarenal distribution of blood flow during ECVE have been obtained with radioactive microspheres. These studies have shown that, in the dog, an intrarenal distribution of blood flow to inner cortical nephrons occurs (*Blantz* et al. 1971; *Stein* et al. 1972; *Bruns* et al. 1974). Surprisingly, in the rat, an intrarenal distribution of blood flow to the superficial cortex following volume expansion has been reported (*Wallin* et al. 1971). However, this discrepancy was attributed to an axial streaming of the microspheres caused by the renal vasodilation and reduced hematocrit accompanying volume expansion. The mechanism of cortical blood flow distribution during acute saline loading in the dog has been examined with microspheres (*Migdal* et al. 1975); their data showed that hemodilution without volume expansion or with intravascular volume expansion alone resulted in a distribution of blood flow to the superficial cortex. This superficial distribution of blood flow was caused by a decreased hematocrit. During saline loading, distribution of blood flow to the inner cortex occurred, which was hypothesized to be a consequence of expansion of the intrarenal interstitial volume.

Two mechanisms have been proposed whereby increased blood flow to inner cortical nephrons could affect renal handling of sodium during volume expansion. The first is the medullary washout hypothesis of *Earley* and *Friedler* (1965a,b). They proposed that saline loading produces an increase in inner cortical and presumably medullary blood flow. This causes a reduction in medullary interstitial hypertonicity, thus reducing the osmotic movement of water out of the descending limb. A larger volume of tubular fluid with the same amount of sodium, but at a lower concentration, would enter the water impermeable ascending limb. Reabsorption of sodium to the same minimal concentration in the ascending limb would result in the delivery of a larger volume of fluid and an increased total amount of sodium beyond the loop of Henle. This alteration would be most marked in the inner cortical nephrons whose loops descend into the medulla.

Another mechanism whereby intrarenal distribution of blood flow may modulate the natriuresis of ECVE is that it could result in a selective decrease in the filtration fraction in inner cortical nephrons and consequent reduction in proximal sodium reabsorption. This hypothesis assumes a direct relationship between peritubular capillary protein oncotic pressure and proximal sodium reabsorption. This hypothesis is based on

experimental observations in several different species. Perhaps the most convincing was a series of experiments in which *Nissen* made use of the unique blood supply to the kidney of the cat (*Nissen* 1968). He calculated the filtration fraction from the postglomerular circulation by comparing the plasma concentrations of inulin and protein in the blood leaving the two venous drainage areas with the concentrations in cortical blood. He observed a greater fall of the filtration fraction in the inner cortex than in the outer cortex after volume expansion. Micropuncture studies in the rat by *Daugharty* et al. (1972), who calculated superficial nephron filtration fraction (SNFF) from systemic and efferent arteriolar protein concentrations, and *Barratt* et al. (1973), who calculated SNFF by means of the antiglomerular basement membrane antibody technique, showed that the filtration fraction was reduced to a greater extent in the whole kidney compared to the superficial cortex during Ringer loading. Likewise in dogs, using the Hanssen technique and microspheres, estimated superficial nephron filtration fraction remained unchanged after volume expansion, but juxtamedullary filtration fraction decreases significantly (*Bruns* et al. 1974).

2.2 Glomerular Filtration Rate

2.2.1 Effect of Changes in GFR

Associated with increases in renal blood flow, several studies have shown that glomerular filtration rate (GFR) is elevated by ECFV expansion (*de Wardener* et al. 1961; *Levinsky* and *Lalone* 1963; *Davidman* et al. 1972). This observation is important as urinary sodium excretion represents the difference between the filtered load of sodium and subsequent reabsorption by the tubules. Therefore, a parallel change in filtered load and sodium excretion seen following volume expansion could, theoretically, explain the rise in sodium excretion seen following volume expansion, without necessarily invoking changes in tubular reabsorption. However, *de Wardener* et al. showed that saline infusion increased urinary sodium excretion independent of changes in GFR. In these studies, GFR was prevented from increasing by simultaneously decreasing the perfusion pressure to the kidney (*de Wardener* et al. 1961). This observation was subsequently confirmed by a number of other investigators who also demonstrated that when ECFV is acutely increased in the presence of fixed adrenocortical activity, sodium excretion increased independently of changes in GFR (*Levinsky* and *Lalone* 1963; *Rector* et al. 1964).

2.2.2 Intrarenal Distribution of GFR

Whereas increases in GFR may not entirely account for the natriuresis of ECVE, the relationship between changes in ECFV and distribution of single neprhon glomerular filtration rate (SNGFR) may be important as it has been proposed that a redistribution of filtrate may modify urinary sodium excretion.

Intrarenal distribution of filtrate before and after volume expansion has been evaluated with a number of techniques. The first is an indirect evaluation and involved comparing superficial SNGFR to total kidney glomerular filtration rate.

The results are conflicting, with some studies reporting equal changes in whole kidney GFR and superficial SNGFR after volume expansion (*Bartoli* and *Earley* 1971; *Mandin* et al. 1971; *Daugharty* et al. 1972; *Davidman* et al. 1972), while others found redistribution of filtrate to outer cortical nephrons (*Gertz* et al. 1967; *Barratt* et al. 1973). These data should be critically evaluated for several reasons. First, an increased distribution of filtrate to outer cortical nephrons was found when a previously punctured tubule was repunctured during acute volume expansion (*Mandin* et al. 1971; *Stein* et al. 1972). In contrast, no change in the intrarenal distribution of filtrate was found when a freshly punctured tubule was utilized. This difference has been atrributed to artifactual alterations in intratubular dynamics in the repunctured tubule due to the high tubular pressure associated with volume expansion, the so-called recollection artifact. However, in sharp contrast, a similar artifact was not noted in other studies in either the dog or rat, in which ECVE caused a proportional increase in both SNGFR and GFR (*Brenner* et al. 1971a; *Schneider* et al. 1972). Second, these results involve comparison of only superficial SNGFR and total kidney GFR, without direct measurement of SNGFR in deep nephrons. It has also been proposed that the disagreement between the results concerning the changes in SNGFR distribution following volume expansion could be explained on the basis of the degree of volume expansion. *Herrera-Acosta* concluded that a 10% body weight volume expansion did not change intrarenal filtrate distribution, whereas a progressive volume expansion to 15% of body weight results in a disproportionate increase in the filtration rate of superficial nephrons in comparison to total kidney GFR (*Herrera-Acosta* et al. 1972).

Another method used to study the distribution of GFR is the ferrocyanide technique. This method involves administering ferrocyanide, which distributes into the extracellular fluid volume and behaves like a glomerular indicator. After injection, the pedicle is ligated, the kidney removed and microdissected. The distance between the glomerulus and the distal front of the precipitated bolus is assumed to be proportional

to the nephron filtration rate. The advantage of this method is that it allows for a more quantitative estimate of the ratio of both superficial and deep SNGFR. Although some studies do not find a change in intrarenal distribution (*Bruns* et al. 1974; *Coelho* 1974), others report an increase in the superficial distribution of SNGFR after either chronic or acute salt loading when the labeled ferrocyanide is given as a pulse injection, i.e., Hanssen's technique (*de Rouffignac* and *Bonvalet* 1970; *Baines* 1973). However, when the glomerular marker is infused, SNGFR increases proportionately in both superficial and juxtamedullary nephrons (*Carriere* et al. 1972; *Coelho* 1973; *Clausen* and *Tyssebotn* 1973; *Poujeol* et al. 1975). This discrepancy was studied by *Charbardes,* and it was concluded that the apparent change in intrarenal distribution during ECVE observed with pulse injection is probably the result of an artifactual streaming caused by the method of administration of the label (*Chabardes* et al. 1974). In addition, a redistribution of SNGFR after acute volume expansion using Hanssen's technique was found in young rats, but not in mature adults (*Baines* 1973). These studies suggest that structural changes with age may modify the response of SNGFR distribution to volume expansion.

Finally, two early micropuncture studies examined directly, by micropuncture of both superficial and juxtamedullary nephrons, the effect of ECVE on the distribution of single nephron filtration rate in these nephron populations (*Horster* and *Thurau* 1968; *Jamison* and *Lacy* 1971). Both studies showed a shift of SNGFR distribution toward the superficial nephrons. However, the physiologic significance of a change in the intrarenal distribution of SNGFR is speculative without knowing whether actual sodium transport is altered in these nephron populations during ECVE. In the acute volume expansion study in which sodium concentration in tubule fluid was measured along with inulin, sodium reabsorption was significantly reduced in superficial nephrons, and did not change in deep nephrons (*Jamison* and *Lacy* 1971). In contrast, a more recent study revealed that delivery of sodium to the bend of the loop of Henle in the deep nephrons was greater than estimates of delivery in superficial nephrons during Ringer loading (*Osgood* et al. 1978).

The hypothesis that the intrarenal distribution of single nephron glomerular filtration rate can affect urinary sodium excretion came from studies by *Barger,* who proposed that because the juxtamedullary or deep nephrons have long loops of Henle, it is possible that more sodium is removed from them than superficial nephrons with their short loop of Henle (*Barger* 1966). Thus, intrarenal distribution of glomerular filtration may modify sodium excretion during ECVE by shifting from deep "salt-saving" nephrons to superficial "salt-losing" nephrons. However, a micropuncture study in the Rhesus monkey, a species with short loops of Henle, shows that the fraction of filtered sodium arriving in the early

distal tubule is similar to values reported for the dog, a species with long loops of Henle (*Bennett* et al. 1968). This would argue against the concept that the length of the loop of Henle is an important factor in the magnitude of sodium transport.

2.3 Physical Factors

2.3.1 Plasma Oncotic Pressure

On the basis of clearance studies in the dog, it was concluded that net fluid reabsorption is determined, in part, by the rate of removal of reabsorbate by the capillary circulation (*Martino* and *Early* 1967). The rate of removal of this reabsorbate is, in turn, dependent upon the net balance of colloid osmotic and hydrostatic pressures across the peritubular capillary wall. Evidence was presented supporting the hypothesis that these "physical forces" play an important role in the decreased proximal tubular reabsorption and increased sodium excretion seen in response to volume expansion.

It was demonstrated that a saline infusion which depressed plasma protein concentration, without increasing arterial pressure or renal blood flow, increased urinary sodium excretion. Subsequent restoration of plasma protein concentration by systemic infusion of hyperoncotic albumin decreased sodium excretion in spite of an increase in arterial pressure and renal blood flow (*Martino* and *Earley* 1967). This observation was taken as evidence that depressed plasma protein is a major determinant of the natriuresis seen with ECVE. Subsequent micropuncture and microperfusion studies, in a variety of experimental conditions designed to assess the importance of plasma oncotic pressure by controlling other physical forces, presented data consistent with the postulate that changes in postglomerular capillary oncotic pressure play a significant role in the regulation of proximal sodium reabsorption (*Lewy* and *Windhager* 1968; *Windhager* et al. 1969; *Spitzer* and *Windhager* 1970). *Brenner,* utilizing both free-flow micropuncture and microperfusion techniques, demonstrated a direct relationship between oncotic pressure in efferent arterioles and absolute reabsorption by the proximal tubule of the rat (*Brenner* et al. 1969b; *Brenner* and *Troy* 1971). The relationship between peritubular capillary oncotic pressure and proximal sodium reabsorption has also been studied in the volume expanded animal. During volume expansion, *Brenner* et al. (1971b) found that capillary microperfusion with hyperoncotic albumin solution increased reabsorption by the proximal tubule and, similarly, *Knox* found that infusion of hyperoncotic albumin solution in the renal artery increased proximal sodium reabsorption in saline

loaded dogs (*Knox* et al. 1973). In micropuncture studies in rats, *Daugharty* compared the effects of iso-oncotic and colloid-free volume expansion on proximal reabsorption (*Daugharty* et al. 1972). In rats in which the filtered load was kept constant by means of aortic constriction, volume expansion with colloid-free (Ringer's) solution resulted in a significant decrease in proximal reabsorption which was associated with a marked reduction in peritubule protein concentration. In contrast, iso-oncotic infusions resulted in no significant fall in proximal reabsorption and little change from control in peritubule protein concentration. *Sonnenberg* and *Solomon,* using free-flow micropuncture, observed that relative to control hydropenic values, extracellular volume expansion with colloid-free Ringer's solution resulted in a significant decrease in proximal reabsorption, whereas following comparable infusions of iso-oncotic fluid, no significant reduction could be detected (*Sonnenberg* and *Solomon* 1969).

Several investigators, however, have failed to find a relationship between peritubule capillary oncotic pressure and reabsorption by the proximal tubule. Specifically, in the absence of saline loading, two studies found no difference in proximal sodium reabsorption between iso-oncotic perfusate versus oncotic-free perfusate (*Rumrich* and *Ullrich* 1968; *Conger* et al. 1973). Further, microperfusion studies showed that reductions in absolute reabsorption induced by saline infusion were quantitatively similar in tubules exposed to the reduced peritubule capillary oncotic pressure and in microperfused tubules with constant peritubular oncotic pressure (*Holzgreve* and *Schrier* 1975). Additionally, *Kuschinsky* et al. compared proximal fractional reabsorption and plasma protein concentration in rats acutely or chronically infused with isotonic saline (*Kuschinsky* et al. 1970). During acute ECVE, proximal fractional reabsorption (FR) was reduced from 61% to 42%, concomitant with a reduced plasma protein from 5.8% to 5.2%. However, during chronic saline infusion, fractional reabsorption (FR) was only slightly decreased to 52%, not statistically different from a control group of animals in which FR was 57%. In these two groups plasma protein concentration was reduced to a greater extent (from 5.9% to 3.6% in the control and chronically infused annimals, respectively). *Ott* et al. investigated the effect of increased peritubule capillary oncotic pressure on sodium reabsorption by the proximal tubule in hydropenic and volume expanded dog (*Ott* et al. 1975). While efferent oncotic pressure during albumin infusion was increased to the same degree in both groups, proximal sodium reabsorption was increased only in the volume expanded animals. The results suggested that ECVE altered the effect of increased peritubule oncotoc pressure on sodium reabsorption by the proximal tubule. It was speculated that increased permeability of the proximal tubule following saline loading

allowed for changes in peritubule capillary oncotic pressure to affect proximal reabsorption. Several studies support a role for altered permeability of the proximal tubule following ECVE. In electrophysiological studies, changes in permeability of the proximal tubule following saline loading in the necturus have been demonstrated (*Bentzel* et al. 1970; *Boulpaep* 1972; *Maunsbach* and *Boulpaep* 1980). Based on these data, it seems likely that increases in membrane permeability are a prerequisite for increased peritubule oncotic pressure to result in increases in isotonic reabsorption by the renal proximal tubule.

Arterial hematrocrit is another physical factor which significantly influences urinary sodium excretion during ECVE. *Knox* et al. found in micropuncture experiments in dogs that acute reductions in arterial hematocrit were associated with a decrease in fractional reabsorption by the proximal tubule, despite maintenance of a normal extracellular fluid volume and no significant change in urinary sodium excretion (*Knox* et al. 1968). Clearance experiments have demonstrated a direct relationship between sodium excretion and blood viscosity during volume expansion (*Schrier* et al. 1970). Further studies in which hematocrit was either increased or decreased in both hydropenic or volume expanded animals, demonstrated that acute changes in hematrocrit significantly affect sodium excretion and renal hemodynamics in both conditions (*Schrier* and *Earley* 1970). Increasing hematocrit during hydropenia or ECVE decreased electrolyte excretion and renal blood flow. Decreasing hematocrit during hydropenia was associated with an increase in electrolyte excretion. The effect of increasing or decreasing arterial hematocrit on proximal sodium reabsorption has been investigated in dogs (*Burke* et al. 1971). It was demonstrated that fractional reabsorption by the proximal tubule can be increased or decreased by acutely increasing or decreasing the hematocrit. It seems likely that the effects of arterial hematocrit are mediated through changes in blood viscosity and subsequent effects on peritubule capillary hydrostatic pressures.

It has also been demonstrated that after anesthesia and surgery animals have a markedly impaired ability to excrete a saline load. *Keck* and coworkers reported that this phenomenon was due to an increased hematocrit, probably due to an increased permeability of the capillaries which results in a large extravasation of protein and fluid into the extravascular space (*Keck* et al. 1973). This observation was confirmed in the rat by *Maddox*, who reported a significant increase in hematocrit accompanied by a large fall in plasma volume following surgery (*Maddox* et al. 1977).

Extracellular volume expansion has also been shown to depress net absorption of sodium and water by rat (*Richet* and *Hornych* 1969; *Humphreys* and *Earley* 1971), dog (*Higgins* and *Blair* 1971), cat (*Gutman* and *Benzakein* 1970), and rabbit (*DiBona* et al. 1974) small intestine.

From studies in the cat, it was postulated that ECVE reduces primarily the lumen to blood sodium flux, analogous to the process of tubular reabsorption in the kidney (*Gutman* and *Benzakein* 1970). On the other hand, two studies in the dog (*Higgins* and *Blair* 1971; *Humphreys* and *Earley* 1971), one in the rat (*Chanard* et al. 1976), and one in the rabbit (*DiBona* et al. 1974) found that ECVE leads to an increase in blood to lumen sodium flux.

The mechanism whereby ECVE depresses intestinal sodium and water transport has been investigated. The results indicate an important role for physical factors in modulating intestinal transport. In vitro studies in dog jejunum showed that when serosal hydrostatic pressure was increased by 2–6 cm H_2O, fluid absorption was stopped, and further increases in serosal pressure resulted in fluid secretion (*Hakim* and *Lifson* 1969). In vitro studies in rats demonstrated that capillary hydrostatic pressure and colloid osmotic pressure exert opposite effects on transjejunal water transport (*Lee* 1973). Further, it has been reported that during ECVE in vivo, the decreased net absorption of sodium is accompanied by an increased permeability of the intestinal mucosa (*Humphreys* and *Earley* 1971). It has also been demonstrated that saline infusion results in a distension of intercellular spaces in the rabbit jejunum (*DiBona* et al. 1974). In addition, in vivo studies suggest that intestinal absorption increases when plasma oncotic pressure is increased by albumin infusion (*Humphreys* and *Earley* 1971). These observations imply that capillary absorption plays a role in intestinal absorption and that permeability changes modulate the net absorption of sodium and water, as may be the case for fluid reabsorption by the renal proximal tubule during ECVE.

2.3.2 Peritubule Capillary Hydrostatic Pressure

Earley et al., on the basis of clearance studies in dogs, concluded that the natriuretic effect of renal vasodilatation, induced either by infusion of vasodilators, plasma or iso-oncotic saline infusion, may in part result from decreased tubular reabsorption as a consequence of the transmission of the increased pressure to the peritubular capillaries (*Earley* and *Friedler* 1966; *Earley* et al. 1966). It also has been proposed that the exaggerated natriuresis of hypertensive patients and rats when given a saline infusion may be due to a direct effect of the increased arterial pressure to decrease fractional reabsorption of sodium (*Papper* et al. 1960; *Buckalew* et al. 1969; *Stumpe* et al. 1970). In addition, a direct relationship between the natriuresis of volume expansion and renal perfusion pressure has been reported from studies in the isolated dog kidney (*McDonald* and *de Wardener* 1965). *Bank* and co-workers, using split-drop techniques in saline loaded rats, reported that when renal perfusion pressure was

lowered to 70–90 mmHg, the reduction in fractional reabsorption which usually occurs in the proximal tubule was greatly minimized (*Bank* et al. 1969). Associated with this finding was a concomitant decrease in urinary sodium excretion. From these results the authors suggested that the changes in proximal reabsorption during saline loading are related to the increase in peritubular hydrostatic pressure. In an attempt to determine whether transmitted increments in capillary hydrostatic pressure are related to decreases in sodium reabsorption during volume expansion, deep intrarenal venous pressure was used as a qualitative index of renal capillary pressure (*Martino* and *Earley* 1968). It was concluded, based on correlations between changes in intrarenal venous pressure and sodium excretion following either saline or plasma infusion, that increases in peritubular capillary hydrostatic pressure inhibit reabsorption. Stop-flow microperfusion with or without combined local microperfusion of peritubular capillaries has shown that, while hydrostatic pressure from the lumen is not an effective driving force for fluid reabsorption, increases in hydrostatic pressure from the contraluminal side markedly inhibit fluid reabsorption (*Sato* 1975). Finally, *Kunau* et al. showed that in rats undergoing a saline diuresis an acute increase in renal perfusion pressure resulted in a marked increase in sodium excretion (*Kunau* and *Lameire* 1976). This natriuresis occurred in the absence of a change in either total GFR or superficial SNGFR. It was also observed that the natriuresis was not the result of an increased sodium delivery from the superficial late distal tubule. This led to the conclusion that it must be related to an inhibition of sodium reabsorption in either the collecting system or in deep nephrons.

2.3.3 Interstitial Pressure

Clearance experiments in dogs demonstrated that administration of dextran in isotonic saline leads to a natriuresis, whereas dextran in glucose does not (*Schrier* et al. 1968). Dextran in saline was associated not only with increased plasma volume, but also with increased interstitial volume, whereas dextran in glucose increased plasma volume alone. From this observation it was postulated that without expansion of the interstitial space, intravascular volume expansion may not be a sufficient stimulus to increase sodium excretion.

Micropuncture techniques have been used to study the respective influence of each of the Starling forces upon proximal tubular reabsorption during hydropenia and volume expansion. The Starling forces, hydrostatic and oncotic pressures across the peritubular capillary wall, presumably mediate capillary uptake and passive backflux into the proximal tubular lumen through changes in renal interstitial pressure. Peritubular capillary uptake is determined not only by the balance of the Starling

forces, i.e., the net reabsorption pressure, but also by the permeability surface area of the peritubular capillary. Thus, changes in either parameter lead to changes in capillary uptake. The relationship between net reabsorption pressure and the permeability surface area of the peritubular capillary (reabsorption coefficient) has been studied in the volume expanded rat with differing results. *Tucker* and *Blantz* measured proximal reabsorption in rats during hydropenia and saline expansion. They concluded that changes in proximal reabsorption correlate best with changes in filtered load (*Tucker* and *Blantz* 1978). *Quinn* and *Marsh* concluded that it was unlikely the reabsorption coefficient was different between hydropenia, Ringer's expansion, and plasma volume expansion (*Quinn* and *Marsh* 1979). They found a strong correlation between absolute proximal reabsorption and net interstitial pressure, which was taken as evidence for a role for interstitial pressure in regulating proximal reabsorption. However, interstitial pressure was directly measured only in saline loaded animals and was calculated for the other conditions. Stronger evidence for a positive correlation between renal interstitial pressure and sodium excretion during volume expansion has been presented by *Marchand,* who observed that reductions in renal perfusion pressure by renal artery constriction prior to volume expansion blocked both the increase in renal interstitial pressure as well as the increase in sodium excretion (*Marchand* 1978). *Kallskog* and *Wolgast* calculated peritubular protein concentration and measured interstitial space pressure (*Kallskog* and *Wolgast* 1973). In saline expansion, the interstitial hydrostatic pressure almost doubled, whereas interstitial oncotic pressure fell to almost zero. They concluded that the peritubular capillary has a large and a small pore system, and further postulated that the effective capillary area increases during saline loading due to expansion of the capillary pores.

Experiments providing evidence that intrarenal factors are the initial determinants of the natriuresis of saline loading were performed by *Fitzgibbons* et al. (1974). As mentioned previously, the natriuretic effect of saline loading has been demonstrated to persist in spite of a reduction of GFR to below preexpansion levels (*de Wardener* et al. 1961). However, *Fitzgibbons* reported that in the rat, reduction of renal artery pressure prior to the initiation of ECVE virtually abolished the natriuretic effect of volume expansion. From these findings, they hypothesized that ECVE can induce a natriuresis only if the kidney has been exposed to at least a transient increase in either interstitial hydrostatic pressure and/or renal plasma flow. Further, the results gave strong evidence against an important role for a reduction in plasma oncotic pressure or a circulating hormone as mediators of the natriuresis. This finding was confirmed in a micropuncture study in the rat by *Osgood* which demonstrated that volume expansion did not decrease proximal reabsorption or cause an

increase in urinary sodium excretion in immediate-clamp rats (*Osgood* et al. 1977). In contrast, comparable aortic clamping 40 min after initiation of volume expansion did not prevent a fall in proximal reabsorption or an increase in urinary sodium excretion. *Marchand* evaluated the effect of reduced peritubule capillary oncotic pressure at reduced renal artery pressure on renal interstitial pressure and sodium excretion in the dog (*Marchand* 1978). He found that reducing renal artery pressure before volume expansion reduces or prevents increases in interstitial pressure and fractional sodium excretion, depending on the magnitude of reduction of renal artery pressure. Studies, also performed in dogs, demonstrated a saline-induced natriuresis, although blunted, even though perfusion pressure was reduced prior to the accompanying volume expansion (*Klemmer* et al. 1978). This study suggested a possible species difference in the renal natriuretic response to volume expansion at reduced renal perfusion pressure between dogs and rats. In order to test the hypothesis that these conflicting results could be due to differences in preexpansion basal sodium excretion, *Lameire* manipulated the dietary salt intake to adjust preexpansion fractional excretion of sodium (FE_{Na} %) to the same level in both species (*Lameire* et al. 1979). Hydropenic or salt depleted dogs showed a small but significant increase in FE_{Na} % following a 7.5% body wt. volume expansion. In the rat, however, a significant natriuresis did not occur unless the rats were salt loaded. These results were interpreted to mean that an appropriate intrarenal environment is necessary in the rat, but not the dog, for volume expansion to result in a significant natriuresis. Further, it was concluded that the natriuretic response to ECVE was not dependent on the moment of lowering the renal perfusion pressure. However, it should be emphasized that in both species, regardless of preexpansion basal sodium excretion, prior reduction of renal perfusion pressure markedly attenuated the magnitude of the natriuresis in response to volume expansion.

2.3.4 Plasma Sodium Concentration

It has been shown that following infusion of hypertonic saline there are increases in plasma sodium concentration, filtered sodium, and urinary sodium excretion (*Kamm* and *Levinsky* 1964). The difficulty in assessing the importance of plasma sodium concentration, per se, as a mediator of the natriuresis following hypertonic saline infusion is that, besides hypernatremia, this experimental maneuver also leads to expansion of the ECFV and a fall in plasma oncotic pressure, all of which could by themselves result in increased sodium excretion. In order to circumvent this problem, *Kamm* and *Levinsky* infused hypertonic saline directly into one renal artery of a dog. Despite a decreased filtered load below that of

preinfusion levels and a comparable volume expansion in the contralateral kidney, the results showed an ipsilateral increase in sodium excretion, which was attributed to an effect of hypernatremia per se to depress net tubular reabsorption of sodium (*Kamm* and *Levinsky* 1965). In order to determine whether a low plasma sodium could depress the natriuretic response to ECVE, *Davis* compared fractional sodium reabsorption in the proximal tubule in normal dogs and in dogs made hyponatremic by chronic administration of vasopressin, water, and ethacrynic acid. Hyponatremic dogs expanded with hypotonic saline had a smaller decrease in fractional reabsorption than normal dogs expanded with an equal amount of isotonic saline (*Davis* et al. 1970). Experiments in dogs, which were infused with equal amounts of sodium chloride per kilogram of body weight as either hypotonic, isotonic, or hypertonic solutions, demonstrated that in spite of the fact that hypotonic saline led to a greater expansion of the blood volume and interstitial space, sodium excretion was significantly less in this group (*Schrier* et al. 1969). Thus, these studies suggest that plasma sodium concentration may influence the natriuretic response to volume expansion.

2.3.5 Preexisting Extracellular Fluid Volume

The effect of the level of the preexisting extracellular fluid volume on the response to acute volume expansion has been studied in the dog and rat. In the dog, animals on a high salt intake and deoxycorticosterone acetate (DOCA) administration have a greater diuretic and natriuretic response to either hyperoncotic albumin solution (*Knox* et al. 1970) or isotonic saline loading (*Higgins* 1971). However, in rats a paradoxical effect of dietary salt intake on the renal response to hypertonic saline loads has been reported (*Ben-Ishay* 1973). Animals on a low salt regimen demonstrated an enhanced diuretic and natriuretic response to hypertonic saline loading compared with rats on a high salt diet. In contrast, studies in dogs maintained on a high sodium intake revealed that they did not have a significantly different natriuresis from those on a low sodium intake, following hypertonic saline infusion (*Diaz-Buxo* et al. 1976). This was also found to be the case whether or not dogs were in positive or negative sodium balance. However, the natriuretic response to isotonic saline loading was smaller in dogs in negative sodium balance compared with those in positive sodium balance. These findings suggest that hypernatremia accompanying hypertonic saline infusion can override factors promoting sodium retention in sodium depleted dogs, but does not further augment sodium excretion in states of positive sodium balance.

2.3.6 Tubule Geometry

A mechanism for glomerular-tubular balance was suggested by *Gertz* and others (*Gertz* et al. 1965; *Brunner* et al. 1966). In this proposal, the rate of proximal tubular reabsorption is thought to be proportional to intratubular volume, as a result of a direct relationship between tubular surface area and reabsorptive rate. Changes in filtration rate result in proportional changes in tubular surface area and subsequently sodium reabsorption. It was proposed that the mechanism by which ECVE suppresses proximal reabsorption was the result of two factors, a decrease in intrinsic reabsorptive capacity and a diminished tubular volume per given level of GFR (*Rector* et al. 1967). However, there is experimental evidence which indicates that tubule geometry does not significantly influence sodium reabsorption. Microperfusion studies, which changed either tubular volume or radius, showed no significant correlation between reabsorption and tubular shape or size (*Morgan* and *Berliner* 1969b; *Burg* and *Orloff* 1968).

2.4 Segmental Sodium Reabsorption

2.4.1 Acute ECVE

There is much evidence from micropuncture studies in the dog and rat to indicate that the natriuresis of acute ECVE is associated with decreased sodium reabsorption by the renal tubule. Numerous studies have demonstrated that ECVE significantly decreases sodium reabsorption by the proximal tubule (*Rector* et al. 1964; *Dirks* et al. 1965; *Cortney* et al. 1965). Because the resulting increase in sodium delivery from the proximal tubule more than accounted for the increased urinary sodium excretion, it was first postulated that the proximal tubule was the sole regulator of the natriuresis of ECVE. This postulate was questioned, however, by studies which demonstrated that proximal sodium reabsorption can be depressed without an ensuing natriuresis, suggesting that a saline load may also inhibit sodium reabsorption in a more distal nephron segment (*Howards* et al. 1968; *Brenner* and *Berliner* 1969; *Knox* et al. 1973; *Knight* and *Weinman* 1977). Evaluation of altered reabsorption during acute ECVE by the "distal" tubule can include the loop of Henle, distal convoluted tubule, and the collecting tubule. The discussion will first focus on evidence for and against the loop of Henle as an important mediator of the natriuresis of volume expansion.

On the basis of studies in humans and dogs utilizing free-water clearance as an index of loop of Henle reabsorption, most (*Eknoyan* et al. 1967; *Stein* et al. 1967; *Leeber* et al. 1968; *Buckalew* et al. 1970b;

Bennett 1973), but not all (*Barton* et al. 1972), report that ECVE suppresses sodium reabsorption in the loop of Henle. The demonstration that the collecting duct has a marked diluting capacity makes interpretation of free-water clearance data difficult. In contrast, micropuncture studies in which sodium reabsorption along the loop of Henle is estimated by micropuncture of both proximal and distal tubules report increased absolute reabsorption after either isotonic or hypertonic saline infusion in the rat (*Giebisch* et al. 1964; *Cortney* et al. 1965; *Landwehr* et al. 1967; *Stein* et al. 1973; *Kunau* et al. 1974) or the dog (*Dirks* and *Seely* 1970). Similarly, microperfusion studies, indicate that sodium reabsorption in the loop increased linearly as a function of the load delivered to it, and that ECVE, per se, does not affect its intrinsic reabsorptive capacity (*Schnermann* 1968; *Morgan* and *Berliner* 1969a).

In many respects, the transport characteristics of the distal convoluted tubule after volume expansion resemble the loop of Henle. *Hayslett,* utilizing the split-drop technique, reported that reabsorption from the distal convoluted tubule is enhanced by saline infusion (*Hayslett* et al. 1967). Many free-flow micropuncture experiments have found increased absolute (*Kunau* et al. 1974; *Knox* and *Gasser* 1974; *Khuri* et al. 1975), and with one exception (*Khuri* et al. 1975), decreased fractional reabsorption along the distal tubule after volume expansion. In addition, micropuncture experiments in which increased sodium delivery to distal tubules was prevented by reduction of renal perfusion pressure revealed that ECVE had no effect on sodium transport along the superficial distal tubule (*Diezi* et al. 1980). These results are in agreement with microperfusion studies which found that the rate of sodium reabsorption paralleled sodium delivery to the distal tubule (*Morgan* and *Berliner* 1969a).

Evidence that ECVE affects sodium transport in the collecting duct has been indirectly evaluated by comparison of sodium delivery in superficial late distal tubules with final urine. Two studies reported diminished fractional sodium reabsorption in the collecting duct after Ringer's infusion when compared with a similar distal delivery of sodium after albumin infusion (*Stein* et al. 1973; *Knox* and *Gasser* 1974). *Diezi* compared urinary sodium excretion in nondiuretic and volume-expanded rats in which late distal sodium delivery was adjusted to similar levels by means of aortic constriction (*Diezi* et al. 1980). Urinary sodium excretion was significantly higher in the volume-expanded animals. The comparison of late distal tubular fluid with final urine to estimate collecting duct reabsorption assumes that homogeneity of nephron function exists with regard to sodium handling. If nephron heterogeneity exists, changes in excretion could be due to either alterations in collecting duct reabsorption per se, or could be due to the admixture of fluid from superficial and juxtamedullary nephrons. In order to differentiate between these

possibilities, *Diezi* performed direct micropuncture of the base and tip of the papillary collecting duct. His results showed that after volume expansion, collecting duct fractional sodium reabsorption decreases and absolute sodium reabsorption increases (*Diezi* et al. 1973). Subsequently, in a similar protocol, *Stein* evaluated papillary collecting duct reabsorption as well as the possibility of nephron heterogeneity, by comparing the delivery of sodium to the end of the distal tubule superficial nephrons with papillary base delivery (*Stein* et al. 1976). They found that the delivery of sodium to and reabsorption along the papillary collecting duct were markedly greater during Ringer loading than in hydropenia. Further, the amount of sodium delivered to the papillary base was greater than the delivery to the end of the distal tubule of superficial nephrons during Ringer loading, suggesting that deeper nephrons deliver more sodium to the collecting duct. Finally, the difference in sodium excretion between Ringer loading and hyperoncotic albumin infusion was due to events occurring between the late distal tubule of superficial nephrons and the base of the papillary collecting duct. Although these findings do not exclude the possibility that ECVE may alter collecting duct transport, they concluded that the natriuresis of Ringer loading was due, in part, to increased sodium delivery from inner cortical nephrons, and that the terminal part of the collecting duct reduces the magnitude of the natriuresis. In a subsequent study, direct micropuncture of the deep nephrons during volume expansion confirmed that delivery of sodium to the bend of the loop of Henle in juxtramedullary nephrons was greater than estimates of delivery in the superficial nephrons (*Osgood* et al. 1978). In addition, micropuncture studies by *Reineck* showed that the net addition of sodium between the late distal tubule and papillary base seen during Ringer loading is abolished if juxtamedullary nephron function is abolished by drug-induced necrosis (*Reineck* et al. 1980). With regard to the finding that in the presence of volume expansion, absolute sodium reabsorption along the papillary collecting duct is enhanced, this is in sharp contrast to the conclusions drawn in several microcatheterization studies that either no net reabsorption or even net addition of sodium occurs along the papillary collecting duct during Ringer loading (*Sonnenberg* 1974, 1975; *Wilson* and *Sonnenberg* 1979). The difference between the findings have been ascribed to technique (micropuncture vs microcatheterization) and experimental protocol. In the studies by *Sonnenberg*, the degree of acute extracellular expansion was markedly greater than in the micropuncture studies and, in addition, urine was reinfused. Urine reinfusion has been shown to be natriuretic (*Harris* and *Yarger* 1976). Accordingly, the differences in results between the micropuncture and microcatheterization studies may be due to the urine reinfusion used in the latter, which in turn may depress sodium transport by the papillary collecting duct.

2.4.2 Chronic ECVE

Chronic ECVE can be produced experimentally by the administration of mineralocorticoids and large amounts of salt in the diet, followed by a return of sodium excretion to control levels. As mentioned previously, this phenomenon is referred to as "DOCA escape". The nephron sites responsible for escape, and the response of the "escaped" animal to volume expansion have been extensively discussed in a previous paper (*Knox* et al. 1980). In brief, superimposed volume expansion in the DOCA-escaped rat reveals that fractional reabsorption of sodium is diminished in the superficial nephron segment containing the pars recta and loop of Henle. Fractional reabsorption of sodium is also decreased in deep nephrons proximal to the bend in Henle's loop. Finally, escape occurs from the sodium-retaining effects of mineralocorticoids when sodium delivery from both superficial and deep nephrons is increased enough to overcome sodium reabsorption by the cortical collecting tubule which is stimulated by mineralocorticoids.

Chronic ECVE is also a feature of models of heart failure and is characterized by the inability to excrete a saline load. *Schneider* evaluated proximal sodium reabsorption in dogs with aorta-vena cava (AV) fistulas to produce high output heart failure (*Schneider* et al. 1971). When these animals are given a saline infusion, the normal inhibition of proximal reabsorption occurs so that delivery from the proximal tubule is markedly increased. However, in these dogs, very little of the increased delivery of sodium appeared in the urine, whereas control dogs had the normal natriuretic response. This observation indicates that sodium transport in some distal nephron segment is altered in response to changes in ECFV. *Stumpe* and associates reported similar findings in rats with AV fistulas (*Stumpe* et al. 1973). Comparing the rats with AV fistulas with normal rats, they found similar delivery of sodium out of the proximal tubule, but enhanced sodium reabsorption along the loop of Henle. In another model of chronic sodium retention, two studies showed that proximal tubular sodium reabsorption was not significantly different in normal animals from animals with thoracic vena cava obstruction after saline loading (*Auld* et al. 1971; *Levy* 1972). *Levy* also found enhanced sodium reabsorption in the loop of Henle in the AV fistula group, again suggesting that sodium reabsorption at this nephron segment accounts for the inability of animals with experimental heart failure to excrete a saline load.

2.5 Hormonal Factors

2.5.1 Renin-Ang otensin-Aldosterone System

It has been propc ;ed that in the antidiuretic animal, glomerular filtration rate is governed y the orthograde flow of tubule fluid past the macula densa and this in turn may be mediated by the renin-angiotensin system (*Schnermann* et al. 1970). This hypothesis comes from the observation that in the antidiuretic state, interruption of flow to the macula densa with proximal tubule micropuncture results in a significantly higher single nephron glomerular filtration rate (SNGFR), compared to the SNGFR measured in the distal tubule beyond the macula densa. These experiments imply that distally measured SNGFR represents the true steady-state filtration rate. However, as stated previously, both proximal SNGFR and whole kidney GFR increase in response to extracellular volume expansion (*Davidman* et al. 1972; *Rector* et al. 1964), and further, it has been demonstrated that the proximal-distal SNGFR difference is abolished during saline diuresis (*Schnermann* et al. 1971). If tubuloglomerular feedback participates in the regulation of filtration rate, one would expect during volume expansion, when distal sodium chloride delivery is elevated, a decrease rather than an increase in filtration rate. *Dev* and co-workers postulated that this apparent discrepancy might be due to variations of sensitivity of the feedback system as a function of salt intake (*Dev* et al. 1974). They found that in rats with a sodium intake of greater than 6 mEq/day, the feedback sensitivity (i.e., proximal-distal SNGFR difference) was abolished. In an extension of these studies, *Schnermann* et al. measured feedback sensitivity by comparing proximal flow rate, filtration rate, and stop-flow pressure in response to loop of Henle perfusion in control rats and DOCA-saline treated rats (*Schnermann* et al. 1975). The DOCA-saline treated rats exhibited a blunted feedback responsiveness compared to controls, accompanied by a significant decrease in juxtaglomerular renin activity. From these observations it was postulated that in animals, either acutely or chronically volume expanded, feedback response is blunted. This impaired feedback accounts for the increase in SNGFR and whole kidney GFR which accompanies ECVE. However, a subsequent study concluded that autoregulation of whole kidney and proximal filtration rate occurred in both sodium depleted and expanded animals in which there was a threefold to fourfold difference in measured renal renin content (*Marchand* 1978).

Studies by *de Wardener* et al. demonstrated that acute ECVE increased sodium excretion depsite the administration of large doses of 9 α-fluorohydrocortisone and reductions in GFR (*De wardener* et al. 1961). These experiments were taken as evidence that increases in sodium excretion

following acute volume expansion were independent of changes in adrenocortical hormone secretion. However, these experiments do not rule out the possibility that acute reductions of aldosterone secretion during volume expansion may be a contributing mechanism for the natriuresis. This possibility was studied by comparing the natriuresis of volume expansion in the intact and adrenalectomized rat. One difficulty of evaluating renal function in the absence of mineralocortocoids is that this maneuver is achieved by adrenalectomy, which usually lowers blood pressure and results in a decreased GFR. Even so, it has been demonstrated that volume natriuresis in response to either isotonic saline or blood volume expansion is similar in intact and adrenalectomized rats (*Cortney* 1969; *Veress* and *Pearce* 1974).

Evaluating the effect of variations in endogenous secretion of mineralocorticoids on the natriuresis of ECVE is difficult. In order to achieve a stimulation of aldosterone secretion, animals are given a low sodium diet and/or administered a diuretic. These maneuvers also contract the ECFV, which in itself affects the renal response to ECVE (*Higgins* 1971). Given the fact that animals administered exogenous mineralocorticoids acutely respond with a diuresis of the same magnitude as untreated ones makes it unlikely that suppression of aldosterone secretion is the primary cause of the saline diuresis. When the ECFV is gradually increased by the chronic administration of mineralocorticoids, an initial retention of sodium is followed by a return of sodium excretion to control levels. This phenomenon is referred to as escape from the sodium retaining effects of mineralocorticoids (*Knox* et al. 1980). The importance of the state of sodium balance in determining the natriuretic response to saline loading has been discussed in Sect. 2.3.5). As mentioned before, an augmented diuretic response to ECVE has been demonstrated in DOCA-escaped dogs. This phenomenon has also been reported in normal humans given 9 α-fluorohydrocortisone (*Strauss* and *Earley* 1959) and in patients with primary aldosteronism (*Biglieri* and *Forsham* 1961). In normal subjects, infusion of isotonic saline suppresses aldosterone secretion, whereas patients with primary aldosteronism fail to do so (*Espiner* et al. 1967). However, both respond with marked natriuresis. This responsiveness of sodium excretion to ECVE reinforces the independence from aldosterone secretion.

2.5.2 Natriuretic Hormone

Several studies have presented evidence suggesting that there is a natriuretic hormone which contributes to the increase in urinary sodium excretion in response to changes in ECFV expansion. Attempts to prove the existence of a natriuretic hormone have employed several different techniques. The first, cross-circulation, was initially used by *de Wardener*

et al. (1961). Cross-circulation involves joining the circulation of two animals via a shunt. One animal serves as a donor and has its ECFV acutely or chronically expanded. In the second animal (recipient) ECFV is kept constant and urinary excretion of sodium is monitored. These cross-circulation studies induce an increase in urinary sodium excretion in the assay kidney, which is taken as evidence of a change in the circulating concentration of a natriuretic hormone. Expansion of the donor animal with saline has been performed by several groups (*de Wardener* et al. 1961; *Johnston* and *Davis* 1966; *Johnston* et al. 1967; *Blythe* et al. 1971; *McDonald* et al. 1967). A number of similar experiments have been performed, except, instead of using saline, the donor animals has been expanded with blood or iso-oncotic albumin and the assay kidney is in situ (*Bahlmann* et al. 1967; *Pearce* et al. 1969) or is isolated and perfused (*Lichardus* and *Pearce* 1966; *Kaloyanides* and *Azer* 1971; *Kaloyanides* et al. 1977). The cross-circulation experiments with saline have been criticized due to the dilution of the blood in the recipient animal (hematocrit, protein concentration), which in itself could result in a natriuresis in the recipient animal. Two groups have addressed this problem in experiments in which they expanded the donor animal with equilibrated blood from a reservoir (*Knox* et al. 1968; *Bengele* et al. 1972). In these animals, as well as those expanded with saline, the sodium excretion in the recipient is usually only a small fraction of that in the donor.

Another approach to detect a natriuretic hormone involves obtaining extracts of plasma, urine or tissue from volume-expanded donors and comparing the natriuresis when injected into bioassay rats with extracts from nonexpanded donors (*Sealey* et al. 1969; *Viskoper* et al. 1971; *Brown* et al. 1972; *Gonick* and *Saldanha* 1975). Again, the ensuing natriuresis is usually modest and sometimes accompanied by an increase in GFR and/or renal blood flow (RBF). These findings raise questions regarding the specificity of the extract, as well as the physiologic significance.

In vitro assays for natriuretic hormone have involved the measurements of ion transport by the isolated frog skin or toad bladder, the transport of *p*-aminohippurate by kidney slices, the activity of kidney ATPase and transport of sodium and potassium by renal tubule fragments (*Bricker* et al. 1968; *Buckalew* et al. 1970a; *Clarkson* et al. 1970; *Nutbourne* et al. 1970; *Katz* and *Genant* 1971; *Gruber* et al. 1980). Many of the findings with in vitro assays have not been confirmed in other laboratories, and the question arises whether the results are relevant to a natriuresis in vivo. The evidence that the natriuresis of ECVE is due, in part, to a natriuretic hormone remains both equivocal and enticing.

2.5.3 Prostaglandins

Saline loading results in an increase in the urinary excretion of PGA and PGE in humans (*Papanicolaou* et al. 1975), as well as significant changes in intrarenal PGA_2 concentration in the rabbit (*Attallah* and *Lee* 1973). Following inhibition of PG synthesis by indomethacin administration, sodium excretion in chronically saline loaded rabbits decreased accompanied by a significant fall of PGA_2 concentration in the outer medulla and papilla. Inhibition of prostaglandin synthesis by either fatty acid deprivation (*Rosenthal* et al. 1974), aspirin (*Susic* and *Sparks* 1975), or indomethacin (*Dusing* et al. 1977b) has been shown to reduce the natriuresis of saline loading. *Dusing* and coworkers studied the effect of prostaglandin synthesis inhibition in ECV-expanded rats on total renal plasma flow and its intrarenal distribution as well as on GFR (*Dusing* et al. 1977a). In ECV-expanded rats, indomethacin had no effect on GFR, but total renal plasma flow was significantly decreased, which was due to a decrease in outer cortical blood flow. To assess the specific sites of enhanced chloride reabsorption in ECV-expanded rats treated with either indomethacin or meclofenamate, *Higashihara* performed free flow micropuncture experiments in the Munich-Wistar rats (*Higashira* et al. 1979). The results showed that inhibitors of prostaglandin synthesis increase chloride transport in the thick ascending loop of Henle, and/or the cortical and outer medullary collecting tubule. Several studies, however, have reported results which showed that inhibition of prostaglandin synthesis results in a significant natriuresis after saline infusion in rabbit, dog, and man (*Oliw* et al. 1978; *Kirschenbium* and *Stein* 1976; *Mountokolakis* et al. 1978). In view of this controversy, the physiologic significance of prostaglandins in mediating the natriuresis of volume expansion is, therefore, not settled.

2.5.4 Antidiuretic Hormone

The possibility that the natriuresis of ECVE is due to a dilution of antidiuretic hormone, thus leading to a diuresis, has been eliminated. Exogenous administration of vasopressin to assure a maximal reabsorption of water has little or no effect on the natriuresis of ECVE (*de Wardener* et al. 1961; *Earley* and *Friedler* 1965a). Further, acute hypophysectomy does not significantly alter the natriuretic response to volume expansion (*Kaloyanides* et al. 1977).

2.5.5 Parathyroid and Thyroid Hormone

Extracellular volume expansion with calcium-free Ringer's solution results in increases in parathyroid hormone (PTH) secretion as a result of a fall

in plasma ionized calcium concentration (*Schneider* et al. 1975). Micropuncture studies in dogs have shown that infusion of parathyroid hormone results in decreased sodium and phosphate reabsorption by the proximal tubule (*Agus* et al. 1971). From these observations one could hypothesize that PTH may play an important role in the natriuresis accompanying ECVE. However, it has been demonstrated that while parathyroid hormone significantly affects the phosphaturia of ECVE, the natriuresis is not dependent on the presence of PTH (*Schneider* et al. 1975).

Holmes and *DiScala* have shown that hypertonic saline infusion in hypothyroid rats resulted in fractional excretion of 45% of the filtered load, whereas no control animal excreted more than 12% (*Holmes* and *DiScala* 1970). This suggests that a thyroid hormone provides a basal support for sodium transport during ECVE.

2.5.6 Neurohumoral

It has been demonstrated that the natriuresis of blood volume expansion is virtually eliminated by high spinal cord section in the dog (*Pearce* and *Sonnenberg* 1965). *Gilmore* and *Daggett* observed a blunted natriuresis to volume expansion with 6% dextran in isotonic saline following chronic cardiac denervation (*Gilmore* and *Daggett* 1966). This was interpreted as being due to interruption of nonvagal afferent pathways from volume receptors near the heart. *Knox* et al. confirmed that cardiac denervation attenuates the natriuresis of saline loading, but found that proximal reabsorption was depressed to a similar degree in response to ECVE as occurs in animals with cardiac innervation (*Knox* et al. 1967). Subsequently, *McDonald* and co-workers investigated the influence of afferent and efferent neural pathways on the natriuresis of ECVE (*McDonald* et al. 1970). High spinal section, which interrupts both afferent and efferent pathways, markedly reduced the natriuretic response to volume expansion. However, cord transection at the T6 level, which preserved thoracic but not abdominal innervation, failed to blunt the natriuretic response. Further, interruption of afferent neural pathways by sectioning nerve roots from C8 to T6, did not impair the natriuresis of ECVE. In this same group of animals, vagotomy also failed to affect the natriuretic response. Similar findings in regard to vagotomy have been shown in response to blood volume expansion (*Pearce* and *Lichardus* 1967). These results strongly suggest that the adrenergic efferent rather than afferent pathways are important in the natriuresis associated with volume expansion in dogs.

Studies have demonstrated that renal denervation in the volume expanded animals leads to an additive natriuresis (*Bengele* et al. 1972; *Bello-Reuss* et al. 1977), which was due to a further decrease in proximal reabsorption (*Bello-Reuss* et al. 1977). Further, low frequency electrical

stimulation of the denervated rat kidney reverses the increase in sodium excretion seen in saline expansion (*Bello-Reuss* et al. 1976). These results are consistent with the notion that a tonic nerve influence on the kidney persists during volume expansion. Studies in the rabbit have, in fact, demonstrated decreased efferent neural traffic during blood volume expansion (*Clement* et al. 1972).

Previous studies have assessed the interrelationship between the catecholaminergic system and ECFV expansion with adrenergic blockade, or measurement of catecholamine blood levels and urinary excretion during acute volume expansion. *Gill* observed an enhanced natriuresis to saline infusion following guanethidine administration (*Gill* et al. 1964). *Alexander* observed that sympathetic nerve activity, as shown by measurement of plasma dopamine β-hydrooxylase and urinary norepinephrine, is suppressed during saline infusion (*Alexander* et al. 1974). Additional studies have confirmed that plasma levels of norepinephrine and epinephrine are reduced during acute saline loading in dogs (*Faucheux* et al. 1977; *Carriere* et al. 1978). These reductions were greater than could be accounted for by hemodilution alone. These data indicate that there may be a direct effect of volume expansion to lower circulating plasma catecholamines, and thereby reduce renal sympathetic tone during volume expansion.

2.6 Response of the Perinatal Kidney to Volume Expansion

When a perinatal mammal is given an ECVE, the result is a smaller absolute and fractional excretion of sodium compared to an adult receiving an equivalent sodium load. This response has been described in several different species (*Aperia* et al. 1972; *Merlet-Benichou* and *de Rouffignac* 1977; *Hurley* et al. 1977; *Bengele* and *Solomon* 1974; *Kleinman* and *Reuter* 1974) and the reasons underlying the phenomenon have been ascribed to several different factors. Among these are rate of glomerular filtration, intrarenal distribution of blood flow, physical and hormonal factors, and inability of the proximal and/or distal tubule to decrease sodium reabsorption in response to a saline load.

The attenuated natriuretic response to volume expansion in the neonate was initially attributed to a relatively small increase in glomerular filtration rate in association with volume expansion. Additionally, a change in the intrarenal distribution of blood flow was postulated to influence sodium handling. The results of studies in newborn and adult dogs, however, show that GFR increases similarly in response to volume expansion in dogs of all ages, and that there is no correlation between the blunted natriuresis and the change in intrarenal blood flow distribution in the newborn dog (*Kleinman* and *Reuter* 1974, *Goldsmith* et al. 1979).

The relationship between changes in physical factors and proximal reabsorption after volume expansion in the neonate is unknown. It has been shown that the oncotic pressure of young rats is lower than that of mature rats (*Allison* et al. 1972). This observation raises the possibility that in the neonate, volume expansion may not decrease peritubular oncotic pressure to the same extent as the mature animal. However, morphological studies demonstrate widening of lateral intercellular spaces during volume expansion in the neonate rat, quantitatively the same as in the mature rat (*Misanko* et al. 1979).

Another possible explanation for the age-related differences in the natriuretic response to volume expansion is elevated blood levels of aldosterone present in the neonate (*Beitens* et al. 1972). It is possible that the neonate kidney is exposed to a greater salt-retaining stimulus due to the amount of aldosterone reaching the kidney, although there is a normal response to volume expansion in the mature animal pretreated with exogenous mineralocorticoids. It has been demonstrated that high sodium intake prior to volume expansion enhances the natriuretic response in puppies (*Steichen* and *Kleinman* 1975).

Results of experiments in newborn dogs demonstrate that the natriuretic response to volume expansion can be restored to adult levels by the administration of oxytocin. Furthermore, the effect was localized to a nephron segment beyond the proximal tubule (*Kleinman* and *Banks* 1980).

Two studies in newborn dogs were carried out to determine whether the attenuated natriuresis to volume expansion is due to enhanced proximal or distal nephron sodium reabsorption. The protocol involved blocking sodium reabsorption beyond the proximal tubule by administration of diuretics, with the rationale being that a change in sodium reabsorption occurring during volume expansion could be attributed to the proximal tubule (*Kleinman* 1975; *Banks* and *Kleinman* 1978). When distal tubular sodium reabsorption was blocked by administration of either amiloride or chlorothiazide and ethacrynic acid, saline expansion increased fractional sodium excretion in the neonate to the same degree as in the untreated volume-expanded adult. These results suggest that the proximal tubule of adult and neonatal dogs respond similarly to volume expansion by decreasing proximal reabsorption, but that sodium excretion in more distal segments of the nephron is augmented in the neonate in response to volume expansion.

2.7 Gastrointestinal and/or Hepatoportal Control of Renal Sodium Excretion

The hypothesis that a sodium ion receptor exists in the gut or portal circulation came from experiments which showed a greater natriuresis when a hypertonic sodium chloride load was given orally rather than intravenously. *Lennane* and *Carey* found that in both rabbit and man, oral sodium loading is followed by a greater natriuresis than intravenous sodium loading (*Lennane* et al. 1975a,b; *Carey* et al. 1976; *Carey* 1978). Because there were no significant changes in either urinary or plasma aldosterone levels between the oral and intravenous groups, it was suggested that the gastrointestinal tract influences renal sodium excretion by a mechanism independent of aldosterone.

However, other investigators have been unable to confirm the hypothesis that a sodium receptor within the GI tract or portal circulation is important in regulating sodium excretion. *Hanson* found no difference in sodium excretion in dogs receiving hypertonic sodium chloride via the GI tract vs an intravenous infusion (*Hanson* et al. 1980). Similarly, results of experiments in rabbits could show no difference in urinary sodium excretion following either a gastrointestinal or intravenous administration of hypertonic sodium chloride (*Obika* et al. 1981). Variations in food intake between the studies may account for the differences seen in dog, rabbit, and man.

It has also been reported that the natriuretic response to a saline load is greater when the infusion is administered into the portal vein rather than into a systemic vein, suggesting that there are osmoreceptors within the portal or hepatic circulation controlling the natriuresis following oral sodium chloride loading. *Passo* reported that hypertonic infusions of sodium chloride into the hepatic portal vein of the cat results in a greater increase in sodium excretion than do comparable femoral vein infusions (*Passo* et al. 1972). Similar results were observed in the dog (*Daly* et al. 1967; *Strandhoy* and *Williamson* 1970). However, using similar protocols, several studies have been unable to demonstrate an enhanced natriuresis following hypertonic sodium chloride infusions into the portal vein of the dog (*Schneider* et al. 1970; *Potkay* and *Gilmore* 1970; *Kapteina* et al. 1978). It is difficult to reconcile the differences reported in the above studies; therefore, experimental evidence for a GI or portal sodium receptor is not conclusive.

Evidence has been presented that the liver may play an important role in renal tubular sodium reabsorption. Enhanced renal sodium reabsorption and impaired renal concentrating ability may be associated with the heptatorenal syndrome (*Shear* et al. 1965). Liver damage produced by ligation of the common bile duct has been used as an experimental model

for the hepatorenal syndrome (*Mullane* and *Gliedman* 1970). It has been reported that dogs with ligation of the CBD have a blunted natriuresis during ECVE, which was attributed to an enhanced proximal and distal tubular sodium reabsorption (*Better* and *Massry* 1972). In an ensuing study, it was noted that ECVE does not elicit a renal vasodilation in animals with CBD ligation, and it was speculated that this may be partly responsible for the blunted natriuresis (*Melman* and *Massry* 1977).

3 Summary

This review summarizes factors influencing renal sodium reabsorption in volume expansion. The influence of renal blood flow and intrarenal blood flow distribution on renal sodium excretion during extracellular volume expansion is unclear. There is no convincing experimental evidence that an increase in renal blood flow per se or a change in the intrarenal distribution of renal blood flow is responsible for the natriuresis of ECVE. An increase in whole kidney glomerular filtration rate has been ruled out as the primary cause of the increase in sodium excretion following volume expansion, and several studies have shown that ECVE is not associated with a change in the intrarenal distribution of GFR. Several investigators propose that the primary variables regulating sodium transport during ECVE are physical factors in the peritubule circulation. There is much evidence to indicate that acute ECVE decreases sodium reabsorption by the proximal tubule. In animals with chronic ECVE, sodium transport along the loop of Henle is decreased. The role of hormonal factors in mediating the natriuresis of volume expansion is controversial. Although current evidence suggests that neurohumoral factors may be important, a role for other hormones, including "natriuretic hormone", is still not settled. In addition, there is no convincing experimental evidence to date to indicate that a sodium ion receptor exists in the gut or portal circulation.

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Hormonal Coordination of the Immune Response

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Contents

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For the last 20 years, hormonal influences on the immune system have been subject to an immense amount of research. The documentation accumulated thus far obviously differs from one endocrine organ to another and in some cases it has been one-sided. About 10 000 publications have been dedicated to the role of the thymus in immunology, whereas references to the role of the thyroid or the gonads are scarce. Immunological work on the adrenal cortex has been considerable, but (with few exceptions) has dealt only with the immunosuppressive influence of corticoids administered in "unphysiological" amounts to intact animals. The concept of a proper hormonal regulation of the immune response, i.e., the role of interactions of several endocrines, under a possible ultimate neurohormonal control, emerged only recently, and its elucidation is largely a matter for future research. The subject of the present review is, among other things, an attempt to indicate possible directions for this research. Thus, the present review has to record immunological and endocrinological documentation. Since a result of these considerations is that the proper endocrinological point of view has for some reason been neglected so far by immunologists (except, of course, for the thymic hormones), this point will be emphasized below. Immunological documentation will be summarized and limited to information necessary to understand present knowledge in endocrinology.

1 The Thymus

As stated above, more than 10 000 references may be found concerning the role of the thymus in immunity [see reviews 216, 218, 255, 329, and 504]. From this tremendous bibliography some valid conclusions can be drawn. A chronological review presents a good idea of the course of this knowledge.

1.1 Immunological Consequences of Thymectomy

The thymus of immunized rabbits contains no antibodies [62] and the thymus of immunized guinea pigs yields no antibodies in the incubation medium [19]. However, thymectomy is followed by a severe decrease in antibody production in weanling guinea pigs injected with *Salmonella* antigens [115, 268], rats [e.g. 24, 525], rabbits [12, 35], and mice [e.g. 15, 252, 256, 261, 362, 469, 493, 557] suffer from a severe immunodeficiency when thymectomized at birth. This is not constant [e.g. 271, 252, 35, 484], but thymectomy in prepuberal mice results in immunodeficciency within a certain delay. The accelerated development of experimental tumors, whether induced by polyoma virus [133, 315], by synthetic carcinogens [368] or by grafting malignant cells [e.g. 421], was explained as an immune deficiency. The rejection of allografts is delayed in mice and rats thymectomized at birth [e.g. 14, 41, 348, 362, 371], while in weanling rats, the rejection of skin allografts is only slightly delayed [123].

The immune deficiency was considered to be the reason for the characteristic wasting of thymectomized mice [e.g. 24] since axenic thymectomized mice do not waste [e.g. 543], and this was generally confirmed [410]. This immunodeficiency of thymectomized animals may be paralleled with the abnormal composition of their immunoglobulins. In these animals, IgM is normal, IgG is deficient, and IgA is absent. Furthermore, sensitized, thymectomized animals fail to perform the switch from IgM production to IgG production during the immune response [see 67 for review].

The immunodeficiency of thymectomized animals (mostly mice) was confirmed by a large and still increasing number of techniques:

1. The plaque-forming cell assay [273]. Plaque-forming cells are severely reduced in number in the spleen of thymectomized mice injected with sheep erythrocytes [e.g. 196, 371].
2. The rosette-forming cell test [e.g. 57]. Rosette-forming cells (with sheep erythrocytes) decreased in number in the spleen of neonatally thymectomized mice [e.g. 29].
3. The graft-vs-host reaction in its in vivo [57, 468] and in vitro form [22]. Spleen cells from thymectomized donors were found to be unable to induce the in vivo [e.g. 134, 347, 505] and the in vitro graft-vs-host reaction [509] (see review of previous documentations on the role in all these reactions [236]).

The (relative) resistance of the immune system in thymectomized puberal mice implies three exceptions:

1. The nude mouse, congenitally deprived of the thymus [549]. This mouse does not reject allografts; its spleen cells are unable to induce

the graft-vs-host reaction and its hemolysin production after sheep erythrocyte injection is conspicuously low. Normal reactions are reestablished in all these tests by a thymus graft [398]. This topic will be discussed below (see Sect. 1.9.1).

2. The dwarf mouse [157, 415].
3. The thymectomized, lethally irradiated mouse, restored with bone-marrow cell transfusion [e.g. 367]. These animals survive both thymectomy and irradiation, but they do not produce antibodies against a large number of antigens, e.g., sheep erythrocytes [127, 367] and skin allograft rejection is delayed [e.g. 361, 515]. This observation developed into a valid and generally accepted experimental design. It may still raise a problem when used to demonstrate the influence of thymic extracts. Indeed, the efficiency of a thymic extract was shown to tend towards zero in irradiated guinea pigs[114].This was not demonstrated by immunological testing; nevertheless, it could explain the inefficiency of thymic hormones in irradiated mice [306].

1.2 Cell-to-Cell Interaction in the Immune Response

Irradiated thymectomized mice, injected with lymphocytes from bone marrow or thymus, produced no antibodies when injected with erythrocytes (as demonstrated with the plaque-forming cell test [367]. Antibody production was normal in similar mice injected with both thymic and bone-marrow cells [e.g. 102]. This could not be observed when the thymic cells were killed by ultrasound [102] or by a specific antiserum. Newborn mice (CBA or C 57b1) were thymectomized, X-irradiated, and injected with sheep erythrocytes. Their antibody production was poor, as demonstrated by the plaque-forming cell test. It could be reestablished by injection of thymic and bone-marrow lymphocytes, but not with thymic cells alone [363, 365, 364].

Newborn thymectomized CBA mice were injected with syngeneic bone marrow and C 57b1 thymocytes together with sheep erythrocytes. Then their spleen was searched for plaque-forming cells. If the test plaques were treated with anti-CBA serum, the number of plaque-forming cells decreased to near zero. Anti-C 57b1 serum did not have a similar effect [363]. Thus, the antibody-producing cells differentiate from the receiver, but the (allogenic) thymus was necessary. In intact X-irradiated mice restoration of the immune response was possible with bone-marrow cell transfusions (plaque-forming cell test); in thymectomized irradiated mice it was not (reviewed in [365]).

CBA mice were thymectomized, X-irradiated, and injected with thymic and bone-marrow cells either from CBA or T_6 T_6 donors (T_6 T_6 thymus

+ CBA bone marrow or the opposite). Their spleen cells were injected into second recipients thymectomized, X-irradiated CBA, and previously immunized against T_6 T_6 mice. In addition, the second recipient was injected with sheep erythrocytes. Three combinations were possible:

1. The second recipient mouse received spleen cells from a donor injected with T_6 T_6 thymus and CBA bone marrow. There was no net increase in plaque-forming cells in its spleen.
2. The second recipient received spleen cells from a donor bearing CBA thymus and T_6 T_6 bone-marrow cells. In its spleen there was an explosive increase in the mitotic rate, but no increase in plaque-forming cells.
3. The second recipient received CBA thymus and CBA bone-marrow cells. Mitosis and plaque-forming cells increased significantly in number [131]. This interaction between at least two cells for immune response was confirmed by numerous in vitro assays [e.g. 43] (see below).

The two (B and T) cells were incubated in two compartments of a double diffusion chamber with sheep erythrocytes added to one compartment only. Antibody production was limited to the B cell compartment, but when B cells were incubated with sheep erythrocytes only, no antibodies were produced [3]. Similar observations were made with *Shigella* antigens [187].

In fact, the cooperation between T and B cells was found to be insufficient [e.g. 13, 363]. Macrophages are also necessary [e.g. 185, 201, 208, 317, 379, 436, see review 282]. Two different T cells seem necessary for this interaction: those killed by antilymphocyte serum (long-lived) and those disappearing shortly after thymectomy [185].

1.3 Role of the Thymus in Immunotolerance

Immunotolerance was first observed in rabbits injected with antigen at birth [246]. This can be induced with repeated small doses or with a single massive dose of antigen. The effect of small doses was enhanced by cortisol injections [100].

Thymectomy was followed by a prolongation of immunotolerance beyond the normal time limit [104, 406, 493]. Thus, tolerance seemed to show just the absence of immunocompetent T cells. Still, these cells may be suppressed by thymectomy or by X-irradiation. In tolerant animals, thymectomy prolonged the tolerance period, whereas irradiation shortened it [101]. Apparently, cells (killed by irradiation) are necessary to maintain the tolerance. Thymectomized, X-irradiated mice bearing

thymus grafts from tolerant donors [264] or infused with thoracic duct cells [99] or spleen cells [243] from tolerant donors became tolerant. This was confirmed in rats [237]. When the (tolerant) spleen or thoracic duct cells were treated with anti-Thy 1 serum prior to the transfusion, this passive tolerance was not induced. Tolerance still does not seem to be specific to T cells. Thymectomized, X-irradiated mice injected with bone-marrow and thymic cells became tolerant when either one of the two cells came from tolerant donors [98]. Enhancement of antibody production or tolerance depends on the ratio of T cells to B cells. Up to a certain limit T cells induce antibody production by B cells; over this limit they suppress it and may induce tolerance [184]. Be that as it may, the importance of the thymus in induction of immunotolerance can be demonstrated.

1.4 Role of the Thymus in Cytotoxic and Graft Immune Reactions

The allograft rejection is a reaction of the cellular immunity. Passive transfer of immunity against allografts was possible by cell transfer but not by injections of serum from an immunized donor [e.g. 372, reviewed 350]. An allograft is rejected at any rate by a normal animal, but receptors of immune cells rejected it as if they had been immunized already by a first graft (i.e., in about half the time required for the rejection of the first graft). Passive transfer of graft immunity by serum was also possible in rabbits [552]. Humoral immunity is supposed to play a part in allograft rejection [see review 478]. Allografts included in diffusion chambers were not destroyed [e.g. 8]. Divergent observations are scarce: lymphocytes of an immunized mouse were included in millipore membranes and implanted into an unimmunized animal. Skin grafts of the strain against which the lymphocyte donor was immunized were rejected by the receptor as second grafts [302, 385]. Drainage of the thoracic duct in rats (i.e., deprivation principally of small lymphocytes) resulted in an significant delay in graft rejection [332]. Thymectomy at birth was followed by a long delay in skin graft rejection in mice [176, 192, 361]. The role of the thymus is essential: X-irradiated, thymectomized CBA mice injected with syngeneic bone marrow and C3H thymic cells normally rejected C 57 B skin grafts but not C3H grafts. Mice injected with anti-Thy 1 serum did not reject skin allografts [428] (see review of previous documentation [542]). Allograft rejection demonstrated the function of cytotoxic cells. Lymph-node cells from rats bearing a skin allograft kill kidney cells from the skin donor strain in vitro [541].

In cytotoxic cells [326], cytostatic activity (demonstrated by the inhibition of thymidine uptake) was distinguished from cytolytic activity

(demonstrated by the release of 51 Cr from the labeled target cells into the medium).

Cytotoxic activity is supported by T cells [514]. It persists in lymphocytes where B cells have been discarded [e.g. 234]. It can be acquired in vitro by cultivation of T cells (from thymus, lymph nodes or spleen) with target cells [see 327]. This differentiation implies DNA synthesis [388]. Cytotoxic T cells are of a peculiar type, characterized by specific antigenic markers (see Sect. 1.7). They conserve their cytotoxic potential for months in vitro in the absence of the specific antigen. They have no "helper" function [see review 136]. This differentiation implies DNA synthesis [e.g. 388].

T cells are sufficient to manifest cytotoxic activity [94]. This observation was not confirmed. Thymocytes (i.e., immature cells) added to the medium, enhanced the cytotoxic activity of (mature) previously sensitized spleen cells from CBA mice against BALB lymphocytes [520]. In fact, cytotoxic activity was observed in a multitude of cells: T cells, B cells, null lymphocytes, and macrophages [93]. Macrophages are required for cytotoxic activity [e.g. 521], but macrophages alone manifest only cytostatic and no cytolytic potency [e.g. 241, 274, 275]. The cytotoxic activity of macrophages may be induced by immunized T cells (immunized T cells conferred a cytotoxic potency to unimmunized bone-marrow cells) [e.g. 240]. Peritoneal macrophages from immunized mice were cytotoxic by themselves (supposed to be influenced by T cells in vivo [324]). It could be shown [84, 323] that the development of cytotoxic activity results from the synergy of two types of T cells, differing only in their antigenic markers (see Sect. 1.8). Cytotoxic activity needs the presence of helper T cells in much the same way as does the differentiation of B cells into antibody producers.

1.5 Suppressor T Cells

Spleen cells stimulated with a mitogen (concanavalin A) added to the medium will reduce the number of plaque-forming cells from spleen cells incubated with sheep erythrocytes [160]. T cells added to the medium induce a decrease in thymus-independent antibodies by B cells [e.g. 205, 206]. Injection of antilymphocyte serum in mice together with a thymus-independent antigen results in an enhanced production of antibodies [e.g. 35]. Mice were rendered tolerant against pneumococcal lipopolysaccharide by a massive injection at birth. Spleen cells of these mice, when added to the medium, will reduce the antibody production of normal spleen cells incubated with the same antigen to zero [152]. In nude mice (which produce thymus-independent antibodies in large amounts), the

injections of thymocytes from their haired littermates together with thymus-independent antigen were followed by a comparatively reduced antibody production [259].

In these experiments the suppression of antibody production appears to demonstrate the activity of a peculiar type of "suppressor" cells. Mouse thymocytes incubated with X-irradiated spleen lymphocytes will develop into suppressor cells and inhibit the ^{3}H-thymidine uptake in a mixed lymphocyte culture. This characteristic is suppressed by pretreatment with anti-Thy 1 serum, suppressor activity is supported by T cells [167] but not by these alone. Immunosuppressor function could be demonstrated in macrophages [565].

The development of thymic cells in either suppressor or helper cells depends on the amount of antigen injected. In the spleen cells of mice, injection of 10^6 sheep erythrocytes induced the development of helper function, whereas injection of 10^9 erythrocytes induced suppressor function. Does this difference show that the same cells possess both potentialities, or does it demonstrate the selective development of one of those cell types?

T cells were incubated with macrophages sensitized to sheep erythrocytes. These cells developed a suppressive influence when added to normal spleen cells, and a helper influence when added to spleen cells deprived of T cells [572].

The normal immune response may thus be the algebraic sum of the helper and the suppressor functions [see e.g. 204, 268]. These functions are supported by different cells. They differ in their antigenic markers. Helper cells bear the Ly 1 marker, whereas suppressor cells are labeled Ly 2, Ly 3 [78, 160, 163]. Two cells are necessary for the development of cytotoxic activity.

It is still open to discussion whether suppressor and cytotoxic activity are of different types or two aspects of the same potential. Cytotoxic and suppressor cells bear the same antigenic marker, but suppressor cells are killed by cyclophosphamide [437] and a suppressor activity against cytotoxic cells could be demonstrated in an allograft test [105]. A distinction could be claimed between suppressor and cytotoxic cells [559].

1.6 Mechanism of the T Cell-B-Cell Interaction

Ever since the notion of T and B cells has been defined, speculation has started on the mechanism of their interaction. As a result hypotheses began appearing in great numbers. Just by the wording, these hypotheses seemed to have provided advanced knowledge, or even better, an illusion of knowledge, but in reality were based on absolutely no facts whatsoever. These hypotheses are obsolete.

Recent investigation has demonstrated that T cells influence the B cells by soluble substances delivered into the medium. Spleen lymphocytes cultivated with sheep erythrocytes develop few plaque-forming cells when previously treated with anti-Thy 1 serum. If T cells previously sensibilized to sheep erythrocytes are added, the development of plaque-forming cells was significantly increased. The same effect was obtained by adding the supernatant of T-cell cultures [455]. In the incubation medium IgG and IgM antibodies were found [455]. Spleen cells from neonatally thymectomized mice (which normally do not differentiate into plaque-forming cells when incubated with sheep erythrocytes) develop plaque-forming cells when incubated with thymic lymphocytes or with the supernatant of their culture [e.g. 146, 235, 526]. Spleen cells from irradiated mice do not develop plaque-forming cells in a culture with sheep erythrocytes. This development is restored by addition of thymic lymphocytes from sensitized donors or by the supernatant of their culture medium [517]. Supernatants of activated T cell cultures induced plaque-forming cell differentiation in spleen cells of nude mice [211]. Mice were grafted with nonisologous skin. The regional lymph nodes were included in millipore chambers and implanted into intact mice of the first graft-bearer strain. The recipients rejected similar skin grafts in the time necessary for a secondary rejection [386].

Thymocytes from mice immunized against sheep erythrocytes were incubated with B cells (spleen cells treated with anti-Thy 1 antiserum) in the two compartments of a diffusion chamber separated by a millipore membrane. The B cells developed numerous plaque-forming cells, even when they came from unimmunized mice or from nude mice [186] or when a dialyzing membrane separated the two compartments instead of a millipore membrane [186]. Supernatants of T-cell culture immunized against *Toxoplasma* induced the production of anti-*Toxoplasma* factor in nonimmunized macrophages [465].

The list of work on this topic is by no means exhaustive. Ten demonstrations were repeated over and over again with reasonably predictable results. Thus, T cells influence B cells by a compound they deliver into the medium. The bulk of T cells can be defined to some degree as a disseminated endocrine gland. Apparently this compound is not specific, however, its production is induced by an antigen. Supernatants from mixed lymphocyte cultures could induce the development of the immune response of B cells against sheep erythrocytes [11, 160, 197, 440, 532]. Divergent observations were reported, however. Mice were immunized against synthetic peptidic antigens. T cells from their spleen were incubated and the supernatant together with the bone marrow was injected into X-irradiated mice. In the spleen of the recipients plaque-forming cells were found to differentiate from the erythrocytes coated with the

same antigens, but not from the normal sheep erythrocytes [384]. This mediator secreted by T cells is probably not unique. Some of these factors are peptides with a mol. wt. ranging around 40 000 [186]; glycolipidic compounds with a similar action were also isolated [566, 569, 584]. The intimate mechanism of their action is unknown, but there is a suggestive parallel. Bacterial lipopolysaccharides mitogenic for B cells enhanced the immune response of B cells against sheep erythrocytes, a thymus-dependent reaction [276].

In fact, several mediators of different chemical nature were described. Ribonucleic acids from lymph nodes of rabbits bearing skin allografts injected into a normal rabbit induced in this animal the ability to reject similar skin allografts as if it had been immunized itself by a first graft [342]. Ribonucleic acids from spleen cells of immunized donors injected into thymectomized mice induced a normal immune response (plaque-forming cell test) in these animals [283]. Allograft rejection accelerated in rabbits injected with ribonucleic acid from lymph nodes of rabbits bearing an allograft [269, 342, 70]. (Still, ribonucleic acids are not supposed to be secreted into the medium.) The "suppressor" incubation medium of mouse spleen cells from donors rendered tolerant by a massive sheep erythrocyte injection contained a ganglioside which showed a suppressor influence in purified form [172].

Soluble mediators probably play a role in the interactions between macrophages and lymphocytes. Macrophages were incubated with an antigen. The supernatant of this culture induced a specific helper cell differentiation when added to the incubation medium of T cells [171].

Cytotoxic action was found to be mediated by soluble products of lymphocytes ("lymphotoxines" [527]). Development of cytotoxic activity in killer cells was found either to be dependent on a soluble product from helper cells [474] or enhanced by the supernatant of a mixed lymphocyte culture ("killing assisting factor" [393]). Supernatant of a lymphocyte culture from mice tolerant to human globulin was found to render mice injected with it tolerant [95]. This (unidentified) compound is secreted by T cells, since pretreatment of the lymphocytes with anti-Thy 1 serum sppressed the reaction. In conclusion, the intricate cell-to-cell interactions, ultimately resulting from the immune response, appear to be mediated by compounds delivered by the different cells into the medium. The intimate mechanisms of these reactions are unknown. Further information on the role of cAMP will be commented on in Sect. 6.1.3. The relation of the cell-to-cell activations mentioned above with the different hormones influencing the immune response awaits further research.

1.7 Distinction of T and B Cells

For many years it was believed that lymphocytes leave the thymus, which led to the conclusion that some of the lymphocytes circulating in the blood or fixed in lymphatic tissues may be of thymic origin. This was demonstrated first by two methods:

1. The consequences of thymectomy on the lymphocyte pool. Thymectomy was followed by lymphopenia and by atrophy of lymph nodes and spleen follicles [reviewed in 116]. In lymph nodes and spleen, the zones affected by this atrophy could be delimited (thymus-dependent zones [see e.g. 399, 400]). These zones include about 70% of the lymph nodes and 20% of the spleen. Lymphocytes in the thoracic duct are, for the most part, of thymic origin. After labeling the thymus in situ with ^{3}H-thymidine, about 0.02%–0.12% of the lymphocytes in the lymph nodes showed the label [533]. Thus, from one test to another the results were very different.
2. The description of thymic lymphocytes of intracellular enzymes differed from those of the lymph nodes' lymphocytes such as alkaline phosphatase [50]. By this test, 10% of the circulating lymphocytes were identified to be of thymic origin, but no such lymphocytes were found in the lymph nodes. This research was placed on solid ground when the antigenic markers specific for the thymic lymphocytes [e.g. 424, 426, 429, 456] were identified, detectable by specific purified antibodies [e.g. 422, 427]. They were different from those directed against the antigenic marker of B cells [424].

These observations made it possible to describe with validity other differences between T and B cells:

The surface of T cells is smooth, that of B cells villous [456]. Drugs such as phytohemagglutinin [e.g. 169] or concanavalin A induce an increased proliferative activity in lymphocytes of thymic origin only. It is possible to conclude from the phytohemagglutinin test that all circulating lymphocytes are of thymic origin [e.g. 145].

T cells and B cells have shown a different mobility in free-flow electrophoresis [e.g. 442, 545, 556]. In fact, this could be expected. In free-flow electrophoresis of normal mouse lymphocytes two populations could be separated, whereas those of thymectomized, irradiated mice, restored with bone-marrow cells, migrated in a unique bulk [441].

In irradiated mice, injected bone-marrow cells migrate in the thymus, the bone marrow, and the lymph nodes. Thymocytes will not migrate into the thymus [193]. More elaborate techniques permitted the identification of several subpopulations in T cells:

1. By the antigenic markers. Within the thymus the Thy 1 antigen is highly concentrated on the surface of cortical lymphocytes, whereas in the medulla and on the circulating lymphocytes the markers Ly 1, Ly 2, and Ly 3 are highly concentrated, and Thy 1 is low [e.g. 84, 481]. The former are killed by an injection of cortisol in milligram dose, whereas the latter survive [65, 297].
2. The cortisol-resistant cells correspond to the mature, immunocompetent T cells, Cortisol was injected in milligram amounts into a first mouse. Its thymus cells were transfused into an X-irradiated mouse together with sheep erythrocytes. Plaque-forming cells in the spleen of the irradiated receptor were about 20 times more numerous than after the transfer of the same number of thymocytes from a normal mouse [106]. Cortisol is known to kill about 95% of the thymocytes [see 147]. The cortisol-resistant cells in the medulla are derivatives of the cortisol-sensitive cortical thymocytes. When applied on the surface of the thymus, ^{3}H-thymidine labeled first the cortical cells, characterized by high density of Th 1 marker on the surface, then later the lymphocytes in the mid-cortex, and lastly those in the medulla, which are known to be cortisol resistant [533].

1.8 Subpopulations of T Cells

Antigenic differences seem to determine the function of the thymic cells. When leaving the thymus, different cells will sttle in the spleen and in the lymph nodes [456].

Research into antigenic markers may identify various subpopulations but of different significance:

1. Different stages in the maturation of T cells
2. Different types of mature T cells
3. Four successive stages can be distinguished.

1. Different stages in the maturation of T cells:

a) The primitive (prethymic) lymphocyte located in the bone marrow. Its surface is smooth (not villous [499]. (Both smooth and villous lymphocytes were found in the spleen of nude mice as well.) It bears a weak Thy 1 [499] antigenic marker which is not dependent on the thymus (it was found in the spleen of nude mice [328, 435]) and in the bone marrow of thymectomized rats [262]. It is assumed that these cells are predetermined to migrate into the thymus when leaving the bone marrow. This seems to result from the observation of *Nakao* and co-workers [387]. Bone-marrow lymphocytes were separated by sedimentation. Those bearing a weak Thy 1 antigenic marker were

thus separated from the so-called stem cells. Injected into irradiated mice these cells, and these only, repopulated the thymus. The stem cells did not. Yet fetal liver cells, i.e., B cells (refractory to anti-Thy 1 antiserum), injected into splenectomized irradiated mice, repopulated in the thymus, lost their antigenic B-cell markers and became sensitive to anti-Thy 1 serum [214].

b) The proper thymocyte, located within the thymus, bearing a high concentration of Thy 1 antigen [e.g. 85, 482].

c) The postthymic immature lymphocyte, still bearing a high density of Thy 1 antigen [482] and a low density of Ly 1, Ly 2, and Ly 3 antigens. They lose the TL antigenic marker when leaving the thymus [297]. All these cells are immunoincompetent. They acquire full immunocompetence at the periphery [482] or in contact with thymic epithelial cells [524].

d) The immunocompetent mature T cells. According to *Stutman* [482], mostly immature immunoincompetent lymphocytes leave the thymus. The few mature T cells within the thymus were said to remain (in grafted thymuses they were still of the donor type one year after the operation [482]). (For a review see [580, 581]).

2. Differences between mature T cells, related to their function:

a) Helper T cells bear the Ly 1 antigenic marker, cytotoxic and suppressor cells the Ly 2 and Ly 3 marker [84, 86].

b) Helper and cytotoxic cells can be separated by sedimentation on a velocity gradient [513]. Suppressor and cytotoxic cells sedimented together and, since they are both Ly^{1-} Ly^{2+}, it is open to discussion whether they are different from each other. The fractionation by free-flow electrophoresis seemed to separate rather the immature port thymic precursors (small, sensitive to cortisol) from the mature T cells (larger, resistant to cortisol [154]). (For review see [205]).

In short, the differentiation of the different lymphocytes involved in the immune response appear to be in some way predetermined and not haphazard (those that do happen to reach the thymus on their migration from the bone marrow and those that do not). Still, all these cells circulate in the same blood, and the hormonal influences they are subjected to may have different consequences from one cell to another.

1.9 Role of the Thymus in Organization and Generation of Immune Response *(H. Wekerle)*

The thymic cortex is a reticular sponge of epithelium-derived cells, which form sparse intercellular contacts via desmosomes. The wide gaps between

the reticular meshwork are densely filled with small to medium-sized lymphocytes, those, particularly close to the reticulum cells, which display frequent mitotic activity. A possibly specialized epithelial cell of the thymic cortex, which might have a central part in T-cell maturation by engulfing certain lymphoid components, will be described below. Principally, the thymic cortex appears to be the location where most of the lymphoid proliferation occurs. Nevertheless, it is not fully clear why this proliferation occurs and what becomes of the cellular progeny which will not leave the thymus.

The thymic medulla is composed of more solid epithelium, which ultrastructurally is distinct from the cortical reticuloepithelial cells. It is still unclear which maturation processes occur in this region. These processes appear, however, to be relatively late in the T-cell developmental pathway, since here most of the T lymphocytes, already immunocompetent, appear to be concentrated. Treatment of mice with high doses of corticoids results in a fast involution of the thymuses, principally of the cortex. Whereas in the cortex practically all lymphoid cells are lost within hours, the fewer medullary thymocytes are mostly preserved. Immunological function tests reveal that the cortisone-resistant thymocytes are immunocompetent, being responsive in lectin-induced mitogenesis, in transplantation, and graft-vs-host reactions.

Generally, a pathway of lymphocyte migration can be observed in postnatal thymuses. Bone-marrow-derived, still incompetent pre-T cells invade the organs across the capsules. Having undergone the developmental steps leading to clonal diversity and propagation, the immunocompetent T cells appear to selectively leave the thymus via the high endothelial postcapillary venules to peripheralize into the lymph nodes and the spleen.

1.9.1 Immunological Functions of T Lymphocytes

Removal of the thymus during embryonic or perinatal stages has grave effects on the morphology and the function of the immune system. Independently *Miller* and *Good* found that thymoprivic animals do not reject foreign tissue transplants. Moreover, removal of the thymus impairs the capacity of the immune system to mount humoral antibody responses of the IgG type and to establish immunological memory. Delayed-type hypersensitivity reactivity and cellular immune responses against viral or related diseases are equally impaired. The depriving effects of thymectomy may be strong enough to prevent orderly growth of developing infant animals. Generalized wasting disease may be the cause of chronic infections by various agents.

The impaired immune function corresponds to similarly grave changes in the lymphoid organ architecture. *DeSousa* et al. investigated the

histology of lymphoid organs from thymectomized mice. They found that the reduction of the organ sizes results mainly from the loss of lymphoid population of distinct fixed areas. The paracortical regions of the lymph nodes and the pericapillary cuffs of the spleeen were depleted. They are truly thymus-dependent areas, since restoration of the thymoprivic animals with transferred thymocytes also restores these areas. In contrast, other areas of the lymphoid organs, such as the primary follicles and the rims of germinal centers, are not affected by thymectomy, and are thus thymus-independent.

1.9.2 T Lymphocyte Differentiation Markers

Thymus-dependent T lymphocytes are morphologically identical with the thymus-independent, bone-marrow-derived B lymphocytes. Both subgroups can, however, be distinguished by several typical markers on their surface membranes. B lymphocytes bear classic immunoglobulin on their membranes. In contrast, T cells lack surface immunoglobulins. In addition, Ig B cells, in most cases bear on their membranes not only Ig, but also receptors for Fc parts of extraneous immunoglobulins, and for C components. Most, but not all, of the T lymphocytes lack these receptors.

Positive T cell markers have been identified following allogeneic immunization. The most prominent among them is the Thy-1 antigen, the original θ specificity. This antigen occurs in two allelic variants, Thy-1.1 characteristic for the AKR/J murine strain, and Thy-1.2 for most of the other strains. Thy-1 antigens are present during T-cell differentiation within the thymus as well as later on peripheral immunocompetent T cells. Moreover, it appears that low concentrations of Thy-1 are present on the membranes of pre-T cells, which have not been processed by the thymus. Except on T lineages, Thy-1 is found in brain tissue, and on certain myogenic cell stages. In contrast to Thy-1 antigen, which is present on most sublines and differentiation stages of T cells, other T-cell markers are typical for limited stages or isolated functional sublines. Thus, they are true differentiation markers. Prime examples for intrathymic developing cells is the TL antigen system, which is not found on normal peripheral T cells, but on some thymus-dependent leukemias. Differentiation markers of peripheral as well as thymic, functionally definable subsets include antigens of the Ly series. Characteristic combinations of Ly-1 and Ly-2.3 antigens, which are genetically unrelated, are found on various T-cell subsets, such as T helpers, T suppressors, T killer cells, and T cells involved in delayed-type hypersensitivity *(vide infra).* Finally, certain T-cell sublines carry on their surfaces components of the major histocompatibility gene complex (MHC) or of associated genes. These include the I-J subregion products of the H-2 (the murine MHC system), which are found

only on suppressor T cells; antigens of the Qa series, which have been recognized only recently, seem to appear only at various mature stages, indluding certain helper populations.

These alloantigenic markers concern only the murine immune system. Analogous antigens are being recognized, however, also in other mammalian species, including man. This holds particularly true for the MHC-associated antigens and Thy-1 analogs. It should be mentioned, however, that human T cells exhibit an unique marker, namely receptors for untreated sheep red blood cells (SRBC receptors), a property which has been highly instrumental in isolating peripheral human blood T and B lymphocytes.

1.9.3 Role of T Lymphocyte Sublines in the Organization of the Immune System

As already mentioned, B lymphocytes are the precursors of the effector cells in the humoral immune response, the plasma cells. The role of the T cells is by far more complex. They include not only precursors for thymus-dependent immune functions, such as killer cells in the graft-rejection response, or in the delayed-type hypersensitivity, but possibly more intriguing is that the T-cell sublines effect the regulation and dosage of immune responses, both on the cellular and on the humoral level. There are T-cell populations which help, or increase, the effector function of either B cells or effector T cell precursors. On the other hand, other T-cell subsets, the suppressor T cells, counteract immune activation either by suppressing the activity of the effector cells directly, or more indirectly, by decreasing the activity of the helper cells required. Obviously, helper and antagonistic suppressor cells are vital components of regulatory systems necessary for fine dosage and self-limitation of a given immune response, as will be discussed later.

Thus, the original concept of clonal selection theories, which imply that a given antigen selects a passively waiting, preexisting lymphocyte clone with complementary receptors, is not totally tenable. Rather, the immune system appears to be a most complicated cybernetic network with many cellular components necessary to elicit one given response and to cause its termination. Obviously, these cells have to communicate with each other. Cellular communications within the immune system can principally occur either via soluble mediators, or via direct cell contacts, supposedly involving complementary receptor/ligand interactions. Both types of interactions have been described in the case of T cells and their reaction partners. Perhaps the best characterized mediator factor is the one emitted by suppressor T cells. It has been shown by *Tada* and his colleagues that suppressor T cells can be activated by antigen in a particular

context to release factor(s) which can suppressively act upon effector lymphocytes. These factors are remarkable insofar as they possess regions complementary to the eliciting antigen. Thus they include genuine antigen receptors. Moreover, the structure of these antigen-binding regions in many respects resembles that of comparable antibodies. The suppressive factors do not express, however, constant protein regions of immunoglobulins. They rather express determinants of the MHC and, specifically, of the I-J subregion, which has already been mentioned as being typical of suppressor T cells. There are claims that helper cells release analogous factors also characterized by antigen-binding regions and determinants of (non I-J subregion) loci of the MHC. These helper factors, quite obviously, would not suppress immune activation but would rather help its initiation and continuation.

In addition to the antigen-specific, T-cell products involved in the regulation of the immune response, numerous antigen-unspecific factors have been identified, which can modulate effector activity. Among them, there are the T-cell replacing factor described by *Schimpl* and *Wecker,* which is thought to act in relatively late differentiation phases of B cell activation. Other factors modulate in vitro mixed lymphocyte reactions and the in vitro generation of T killer cells, and others appear to promote growth of single T lymphocytes to clones.

Direct intercellular interactions are not as accessible as humoral interactions in terms of experimental investigations. There are, however, some stages of the ongoing immune response which undoubtedly depend on intercellular contact and recognition. The first stage of any immune reaction is recognition of the immunogen by the responding, complementary lymphocyte. A plethora of experimental data indicate that in the case of T cells mere binding of antigen to the complementary receptor regions is necessary (and this is true for many, if not all, T-cell sublines), however, it is by no means sufficient for recognition leading to T-cell activation and differentiation. The studies of *Zinkernagel* [587, 588] and *Doherty,* of *Shearer,* and of *Rosenthal* and associates showed that T cells have to recognize "their" antigen in the context of determinants of the self MHC. Thus, a virus-specific effector T cell would only recognize "its" virus when it is presented on a somatic cell of the same organism, thus posessing the same MHC antigens as the T cell. The same is basically true for the recognition of soluble or particulate antigen. Any antigen must be presented by an autologous cell, and this is done exclusively by the macrophage or by its functional sublines. It is still unclear how the associative recognition of antigen plus self recognition is effected by the T cells. The following principles seem to be valid. T-cell recognition aimed at eliminating a membrane-expressed (foreign) antigen, such as virus-infected cells, is associated with corecognition of the classical transplantation antigens

(H-2K/D determinants in the mouse, HLA-A/B/C in humans). T-cell recognition, which leads to regulation and dosage of immune responses, in contrast, involves recognition of Ia determinants, which are equally coded for in the MHC, but which are predominantly expressed on lymphoid cells and macrophages in the body.

The requirement of Ia antigen identity is also marked in reaction stages subsequent to antigen recognition. They may be the most important examples of contact interactions in the immune system. Ia antigen compatibility is apparently required for various stages of interactions between helper T cells and B cells as well as between suppressor cells and their respective targets, i.e., helper T cells and B cells.

1.9.4 Generation of Antigen Diversity in the Thymus

One of the most intriguing problems in cellular and molecular immunology is how the structural diversity of the antigen-complementary lymphocyte surface receptors is generated. For decennia, the core question was whether receptor diversity is precoded firmly in the genetic information ("germ-line hypothesis") or whether it is rather the result of somatic mutation. Recent experimental evidence, stemming from the work of *Tonegawa* et al., appears to favor a compromise. It appears now that most of the structural information of receptor amino acid sequences is, indeed, contained within the transmittable genetic information. It is, however, probably arranged in relatively small sequence fragments, which are somatically recombined to give rise to the structural diversity underlying the clonal complementarity.

It is not known how and where exactly the cellular events, basic to molecular diversification, are happening. In the case of T cells, it is very probable that generation of diversity occurs within the thymus. This may be particularly evident in generating MHC determinant corecognition by developing T cells. *Jerne* has postulated that incompetent lymphoid precursors, which express receptors for self antigens, enter the thymus and start to interact there with their complementary "self" MHC determinants present in thymic stromal components. As a consequence, the primitive cells start to divide. In the course of proliferation, selective mutations were thought to alter the self-recognition, T-cell receptors to receptor structures complementary to some foreign structure. Indeed, *Zinkernagel* et al. reported of experiments which support part of this hypothesis. Their experiments suggest that the capacity of T cells to corecognize self MHC determinants along with the foreign antigen structures is acquired while in the thymus, probably following a selective interaction with the thymic stroma. *Wekerle* and his colleagues very recently have found a new thymic reticular cell type, which could be involved in

that particular stage. These cells, thymic nurse cells (TNCs), are of epithelial origin as indicated by their ultrastructure and have the capacity to be invaded by viable T lymphocytes [585, 586]. Invasion appears to be the consequence of (self) recognition. Subsequently, the incorporated cells start to divide within their nurse cell. When the engulfed lymphocytes are completely surrounded by internal epithelial membranes, which have several specializations indicative of secretory activity, it is possible that the particular microenvironment may lead to guided differentiation. This could result either in receptor diversification or in ramifications of the specialized, functional T-cell subsets. Thymical hormonal factors could act particularly stringently in this environment. (In addition to receptor diversification, specialization into various functionally specialized T-lymphocyte subsets is assumed to occur within the thymus. It should be noted that incompetent precursor cells enter the organ and that their immunocompetent progeny leave it finally as specialized subsets, as indicated by functional tests as well as by suface marker analyses.)

1.10 Substitutive Therapy for the Thymoprivic Condition

Restoration of normal reactions in thymectomized animals (and in nude mice) was attempted by several methods and demonstrated by several tests. These tests are not equally significant. The most convincing is the survival test in animals known to waste and die following thymectomy, such as mice, hamsters, and guinea pigs. Certainly, this is only a basic test, and its results must be rechecked with specific immunological tests. However, thymic preparations previously tested on the survival of thymectomized animals acquire thus an outstanding validity when compared to those not tested in this way. This distinction is always made below.

1.10.1 Thymic Grafts

Wasting was prevented in thymectomized mice by thymic grafts [134] and their immune functions were restored [134, 486]. These mice rejected skin allografts normally [314, 318, 361], and their reactivity towards sheep erythrocytes (plaque-forming cell test) was restored [7, 130, 314]. This experiment was repeated over and over again with similar results. Conversely, immunocompetence of spleen cells in thymectomized rats (0 previously) was restored following their perfusion through a thymus [80]. The accelerated development of benzopyrene-induced skin tumor in thymectomized mice was abolished by thymic grafts [358]. Thymic grafts were able to restore the immunocompetence of thymectomized animals when enclosed in diffusion chambers [7, 212, 448, 487, 549].

Incubation of mouse thymocytes with thymic epithelium resulted in an enhancement of their activity in a mixed lymphocyte culture [379]. This was tested by numerous methods [7, 320, 346].

Grafts of thymuses deprived of thymocytes (by previous X-irradiation or by previous incubation in vitro [e.g. 252, 521, 524] were similarly active. In nude mice, thymic grafts, enclosed in diffusion chambers, induced a decrease in the lymphocytes bearing a (weak) Thy-1 marker [435].

These experiments support the supposition of a hormonal influence of the thymus.

1.10.2 Transfusion of Thymic or T Cells

Wasting of thymectomized mice was prevented by transfer of thymocytes [e.g. 165, 553]. These animals recovered normal immune potency. Similar results were obtained with transfer of spleen cells [e.g. 363, 486]. In prepuberal thymectomized and X-irradiated mice this technique was extensively used to restore immune functions [e.g. 363, 127]. The increased sensitivity to polyoma virus in thymectomized mice was suppressed by transfer of spleen cells [313]. Every study has reported that the results of this therapy are transitory, although the influence of thymic epithelial grafts lasts as long as the graft itself. If the effect of thymocytic transfer results from the influence of thymic hormones, this could indicate that thymocytes carry the hormone but do not produce it themselves.

1.10.3 Cell-Free Thymic Preparations

The ideal way to attempt substitutive therapy in thymectomized animals was to use the supernatant of thymic epithelial cell cultures. However, to obtain this compound in amounts sufficient to prevent wasting in dozens of thymectomized mice is wishful thinking. This technique was limited to miniaturized tests under cell-culture conditions.

A single injection of thymic culture supernatant accelerated the development of lymphatic organs in newborn mice [351].

The (weakly) Thy-1 spleen cells of nude mice acquired an increased reactivity in a mixed lymphocyte culture, when previously incubated on a monolayer culture of thymic epithelium [445]. The supernatant of thymic epithelial cultures, added to the incubation medium of spleen cells from nude mice gave them the capacity to differentiate into plaque-forming cells [307]. Mice were immunized against rat fibroblasts, thymectomized, and X-irradiated. Spleen cells from these mice, when injected into the hind paw of rats, induced only a very weak swelling of the popliteal lymph nodes of the recipient. The swelling of the lymph nodes was significantly enhanced when the spleen cells were previously incubated

with the supernatant of a monolayer culture of thymic epithelium. After incubation with the supernatant of thymus culture, spleen cells from nude or thymectomized mice induced a graft-vs-host reaction and a sensitivity to concanavaline A. This was not the case with spleen cells from intact normal mice [324, 356]. Thus, the supernatant of a thymus epithelial culture proved to exert a specific influence on lymphocytes. It acted inasmuch as it substituted for the thymus.

1.10.4 Thymic Extracts

The simplest cell-free preparation is the supernatant of homogenized thymic tissue washed by an appropriate solvent (water, saline etc.) and separated by centrifugation. The first preparations were tested for their stimulatory influence on lymphatic cell proliferation in newborn animals [242, 351] and in rats made leucopenic by irradiation [83]. Injected into intact mice, the supernatant of a thymus homogenate induced an increase in labeled thymidine and glycocoll uptake of the lymphocytes in the thymus, the lymph nodes, and the spleen. Immunological tests with similar compounds are few. Supernatants of homogenized Sprague-Dawley rat thymus, injected into neonatally thymectomized Sprague-Dawleys bearing Wistar rat skin grafts, accelerated the rejection from 60 days (in untrated rats) to 12 days [81]. A crude aqueous extract from acetone-defatted calf thymus enhanced the antibody productions of irradiated rabbits [357]. A crude extract of rabbit thymus prolonged the survival and delayed the tumor development in thymectomized mice grafted with Lewis sarcoma [463].

Experimental testing of crude organ preparations is rarely satisfactory. The action of these preparations is always the expression of a sum of the actions of several compounds. This sum may be algebraic: protein fractions isolated from the thymus were shown to inhibit lymphocytic proliferation [289] and various aspects of the immune response [87, 381, 405]. A dialyzable, nonpeptidic (pronase-resistant) immunosuppressive fraction could be isolated from calf thymus by precipitations with mercury nitrate [560]. The description of several extraction and fractionation methods made the use of crude thymic extracts very soon obsolete.

1.10.4.1 Purified Extracts

Commonsense tells us that several truths must be emphasized from a biological point of view before discussing this topic.

1. An organ extract expected to contain a hormone is worth as much as the bioassay tests proposed for its activity. This truism is valid as long as the hormone is not obtained in a pure state and its composition is not known exactly.

2. One test is no test. Several tests may be valid and yet in contradiction to each other. They may have different meanings.
3. There is only one way to distinguish a hormone from a specific cellular constituent. By definition a hormone is secreted. This can be demonstrated by two experiments:

a) The hormone (or its characteristic activity) is present in normal animals in the corresponding gland and elsewhere.
b) Following extirpation of the gland, the hormone (or its activity) disappears entirely.

There are two sorts of biological tests:

a) Global tests. The typical example is the survival test, if available (as for the thymus in mice, hamsters, and guinea pigs). This, however, is only a first step, just demonstrating indubitably the existence of the hormone.
b) More elaborate tests which elucidate the mechanism of the hormone action considered. These tests attempt to define which functions are lost following, e.g., thymectomy, and which thymic extracts are able to restore these functions. These tests make much use of isolated organs or cells. It is obvious that these tests performed in vitro are more convincing if the cells used come from thymectomized animals.

1.10.4.2 Peptidic Extracts

Extracts Known to Ensure Survival of Thymectomized Animals: Thymosin (A.L. Goldstein and co-workers)

Thymosin was obtained from a saline thymic extract by heating it to 80°C (precipitate discarded) and by successive precipitation with acetone and ammonium sulfate. Further purification was attained by successive chromatography on Sephadex, carboxymethyl cellulose, and diethylaminoethyl cellulose [222, 224, 258, 217, 218]. A homogeneous peptide was thus obtained and its amino acid sequence determined [571]. Several consecutive reviews of this work were published [217, 218]. Of the thymextomized mice injected neonatally with thymosin, 70% survived with no obvious disorders, whereas only 34% of the untreated mice survived. The dose injected was thus just below the optimal dose (which perhaps would restore the mice 100%). In these mice the blood lymphocyte count (decreased following thymectomy) was restored to (about) normal. Thymosin seems to exert a stimulatory growth influence on lymphocytes. It hastens the regeneration of lymphatic tissue following X-irradiation [220] or cortisol injections (in enormous amounts [450]).

Thymosin was injected into neonatally thymectomized C57 B1 mice at 1 mg daily (a partially purified compound). Spleen cells from these mice were injected into newborn BALB mice in which they induced graft-vs-host

reaction, whereas spleen cells from untreated thymectomized mice did not. Similar doses of spleen extracts injected were found to be inactive [315]. Since the spleen contains ten times less hormone than the thymus [121, 329], this dose was probably insufficient. Injected into thymectomized mice thymosin enhanced the proliferation of their spleen cells in mixed lymphocyte culture [423].

Thymectomized mice injected neonatally with thymosin normally rejected skin allografts [219, 243]. They rejected normally, after a transitory growth, sarcomas induced by Moloney virus [558], whereas there was no rejection of those tumors in neonatally thymectomized untreated mice. Conversely, injections of an antithymosin antiserum induced a significant delay in allograft rejection [248]. In thymectomized mice injected with antithymocyte serum thymosin had no effect on tumor allograft rejection [423].

The influence of thymosin on humoral immune response in thymectomized mice was less significant [219, 248, 483]. (Nevertheless, thymosin injections could not restore the ability of neonatally thymectomized mice to reject allografts or their spleen cells to induce a graft-vs-host reaction in newborn mice [483].) In thymectomized, X-irradiated rats (900 rad) reconstituted with isologous bone-marrow thymosin injections had no influence on lymphatic tissue regeneration and antibody production [306]. We should recall that in irradiated guinea pigs the influence of thymic hormone tended toward zero [111].

In nude mice, thymosin injections induced an increase in the number of plaque-forming cells after an injection of sheep erythrocytes [63]. When incubated with thymosin, spleen cells of nude mice developed plaque-forming cells [11]. Incubation of bone-marrow lymphocytes with thymosin resulted in the appearance of antigenic markers specific for T cells on the spontaneous rosette-forming cells [30]. The enzyme terminal deoxynucleotidyl-transferase, characteristic for thymocytes, was induced in bown-marrow cells of thymectomized or nude mice [401].

In humans suffering from immune deficiency thymosin was largely used. The first case report (as far as we know) was that of a girl suffering from almost constant recurrence of infections constained for years with antibiotics. She was unable to develop positive cutaneous reactions after an infection *(Candida sepsis)* or vaccination (mumps) or to produce antibodies when challenged with various antigens. Spontaneous rosette-forming cells in her blood were significantly rare. All these abnormalities were suppressed by repeated thymosin injections [529]. Thymosin therapy for immunodeficient patients resulted in an improved general condition, an increased blood lymphocyte count, and in an increased number of rosette-forming cells [225, 529]. In cancer patients similar results were obtained on lymphocyte count and the number of rosette-forming cells.

"The clinical response must be considered anecdotical" [217, 447]. Incubation with thymosin induced the development of antigenic markers in human lymphocytes (from healthy subjects and one cancer patient [503]). Incubation with thymosin of circulating human lymphocytes resulted in an increase in the number of rosette-forming cells [528]. The same influence was found with fetal calf serum [82]. The authors consider this observation as (primary) evidence for the delivery of thymosin into the circulation. Still, serum of nude mice has shown the same influence (*T.D. Luckey,* unpublished communication).

Thymosin Activity Tested In Vitro. Thymosin was added to the incubation medium of lymphocytes. The cells, thus treated, were tested for their degree of differentiation and maturation. Incubation with thymosin induced the appearance of antigenic markers of thymocytes in mouse bone-marrow lymphocytes, in lymphocytes from nude mice or from 14-day-old fetuses [295]. The maturation of spleen cells from mouse fetuses and newborn mice was hastened (as demonstrated by their ability to induce graft-vs-host reaction and their behavior in mixed lymphocyte culture) by previous incubation with thymosin [108, 221]. Bone-marrow cells from newborn mice were incubated with thymosin and injected into thymectomized, X-irradiated mice together with normal bone-marrow cells. In the spleen of the recipients, normal numbers of plaque-forming cells were found, just as if they had received bone marrow and thymocytes [359]. In bone marrow incubated with thymosin, the number of cells killed by an (brain!) anti-Thy 1 antiserum increased [358, 503]. Lymphocytes from nude mice, from fetal mouse liver or from bone marrow differentiated into T cells as evidenced by specific antigenic markers, by their ability to ensure helper function and to form T-cell rosettes with sheep erythrocytes [451]. Thus, thymosine was proved to be active in thymectomized and in congenitally thymoprivic mice. This is demonstrated by the survival test and by a large range of immunological tests.

Extracts from a culture of thymic epithelial cell, prepared with the thymosin method, showed immunostimulant properties similar to those of thymosin [563]. But it must be said that the survival test was performed only once with a very crude preparation. The last steps of purification were only tested by the rosette test, and the reliability of these results may be questionable (*Luckey* and co-workers).

Thymic Humoral Factor (Trainin and co-worker)

Preparation [301, 508, 506]. A centrifuged, thymic homogenate in 0.1 M phosphate buffer at pH 7.4 was fractionated by successive dialyses (discarding by the first molecules of 120 000 and by the second molecules of 6000). The second dialysate contained the hormone in purified form. Precipitation by ammonium sulfate was not carried out.

Injections of the thymic humoral factor reduced the incidence of wasting in neonatally thymectomized mice to 20% (from 60% in untreated mice [510]). This treatment prevented thymoprivic atrophy of lymphatic tissue and the decrease in the number of circulating lymphocytes. Thymectomized mice thus treated rejected skin allografts normally [507, 505].

The authors made much use of the in vitro graft-vs-host reaction test [22]. Spleen cells from neonatally thymectomized mice were found unable to induce the growth of an allogenic spleen fragment when added to the medium. This capacity was restored by repeated injections of thymic humoral factor [505]. Spleen cells from thymectomized mice acquired the graft-vs-host ability when incubated with thymic humoral factor [509]; such treatment also increased the survival rate of thymocytes when cortisol was added to the medium. Thymocytes incubated with thymic humoral factor induced the in vitro graft-vs-host reaction. In other words, they behaved like the few mature T cells present in the thymus, which are also resistant against cortisol [511]. This indicates that thymic humoral factor is involved in some way in the maturation process of thymocytes.

This supposition was substantiated by further experiments. Prepuberal mice were thymectomized, X-irradiated, restored with bone marrow, and injected with sheep erythrocytes. Spleen cells from these mice did not differentiate into plaque-forming cells, even when the mice received thymic humoral factor injections. The normal reaction was restored when the mice received injections of thymocytes together with the hormone, or thymocytes preincubated with the hormone [439]. Mice were injected with anti-lymphocyte serum. Spleen cells from these mice did not induce the in vitro graft-vs-host reaction, even when thymocytes were added. The reaction was restored when thymocytes and thymic humoral factor were added together [327]. Lymphocytes from thymectomized mice reacted poorly in mixed lymphocyte cultures. Normal reaction was restored when thymic humoral factor was added. The addition of thymic humoral factor had no effect on lymphocytes from thymectomized, X-irradiated mice, unless thymocytes (inactive by themselves) were added together with the hormone. Thymic cells from a mouse, previously injected with 5 mg of cortisol, enhanced the mixed lymphocyte reaction by themselves, but in this system thymic humoral factor had no additional influence [516]. This indicated:

1. That the target of thymic humoral factor as the immature thymocytes, in which it induces the maturation to T cells.
2. That the cells able to respond to thymic humoral factor are in some way predisposed to do so. (Could this explain the inactivity of the thymic hormone in irradiated animals mentioned above?)

Mouse fetal liver lymphocytes, i.e., B cell precursors (only?), incubated with thymic humoral factor, induced the in vitro graft-vs-host reaction [213]. Yet, spleen cells from thymectomized mice preincubated with thymic humoral factor did not induce the in vitro graft-vs-host reaction [486], and thymic humoral factor did not induce transplantation immunity in nude mice [408; see also 579].

The mechanism for the action of thymic humoral factor is not known.

Homeostatic Thymic Hormone (Comsa and co-workers)

Preparation [see 113], from a crude acidic extract (H_2SO_4 M) fractionated successively by ammonium sulfate precipitation and isoelectric precipitation in 60% alcohol solution (pH 7 for impurities, pH 6.2 for the active fraction) a homogeneous compound was obtained by successive chromatography on Sephadex and hydroxyapatite (elution with potassium phosphate buffer at pH 6.75 in a molarity gradient).

Wasting in thymectomized guinea pigs was prevented by using partially purified thymic extract and a purified fraction of this same extract [see 113]. It was a complete success, since all thymectomized guinea pigs thus treated survived in good condition, whereas only 60%–70% of neonatally thymectomized mice were preserved from wasting by administering thymosin or thymic humoral factor injections. Wasting after thymectomy is less frequent in guinea pigs (50%–60%) than in mice (70%, according to *A. Goldstein* and co-workers). In rats thymectomized at 30 days, injections of homeostatic thymic hormone prevented the consequences of thymectomy on the endocrine glands and on lymphatic tissue [see 113].

Circumstantial evidence was obtained to support the supposition that this hormone is secreted. It was found in the thymus, in the lymph nodes, and in the spleen or normal prepuberal rats, in the ratio 10:3:1 [124].

Three days after thymectomy, it disappeared from the lymph nodes and the spleen [121]. Its secretion is influenced by the adenohypophysis, the adrenal cortex, the thyroid, and the gonads (see Sect. 6.2).

Immunological tests performed with this compound are scarce. In infantile thymectomized guinea pigs, it restored the antibody production against HO *Salmonella* antigen [115]. In thymectomized and hypophysectomized rats it restored the influence of hypophyseal growth hormone on antibody production [125] and on allograft rejection [123]. The acceleration of allograft rejection, induced by corticotropin, was suppressed by a simultaneous injection of thymic hormone ("The thymic hormone is immunostimulant and immunosuppressive. It all depends on the hypophyseal hormone it has to face") [123].

Thymosin or homeostatic thymic hormone delayed the death of mice injected with a supernatant of *Yersinia pestis* culture [141]. This may indicate an immunostimulant influence, among others.

With the allograft rejection test it could be seen that the thymic hormone is only needed for the primary reaction: Skin allografts were rejected by normal rats within 7.1 ± 1.0 days and by thymectomized rats in 9.8 ± 1.3 days. Second grafts implanted in those rats were rejected in 4.6 ± 0.5 and 4.4 ± 0.4 days, respectively.

The hormone of *Bernardi* and *Comsa* exerted a chemotactic influence on lymphocytes. However, it cannot be said on which one it was [125]. This has been presumed [349, 245, see review] with no direct evidence.

Considering the intricate interactions between the homeostatic thymic hormone and other hormones, this topic will be resumed below (see Sects. 2–6).

Thymic Polypeptidic Hormones Not Tested with the Survival Tests

Thymopoietin (G. Goldstein and co-workers)

Preparation. An extract with 0.1 M ammonium carbonate was fractionated by heating to 70°C, dialyzed, and successive chromatographies on Sephadex and hydroxyapatite and polyacrylamide electrophoresis were carried out [228]. By this method two different homogeneous fractions were obtained, both active in *Goldstein*'s test. Both peptides were obtained in a pure state and their amino acid sequence was determined [461].

The starting point of this work was the observation of an accelerated neuromuscular transmission in thymectomized animals, which can be prevented by thymic grafts [230]. Conversely, the neuromuscular chronaxy was found to be decreased in thymectomized rats [420]. From calf thymus, an extract could be prepared which, by repeated injections, induced a neuromuscular block in guinea pigs [226]. The neuromuscular disorder induced by this compound (named thymin, now called thymopoietin) is described in detail in [231]. The guinea pigs served as a basis for bioassay using thymopoietin.

Culture of bone-marrow lymphocytes or of the B-cell fraction from mouse spleen lymphocytes with thymopoietin added resulted in the appearance of TL and Thy 1 antigenic markers [42, 296]. Spleen lymphocytes from nude mice treated with thymopoietin acquired the antigenic markers TL and Thy 1 and developed a helper influence toward B cells, as shown by the plaque-forming test [450]. In the B-cell fraction of mouse spleen lymphocytes, the addition of thymopoietin to the medium induced the appearance of TL antigenic marker [43]. The cells containing terminal deoxynucleotidyl-transferase from mouse bone marrow are lysed by anti-Thy 1 antiserum after pretreatment with thymopoietin (in other words, they differentiated into prothymocytes [232]). In a one-way mixed mouse lymphocyte culture, thymopoietin induced an increase in the cyclic AMP content [488]. In thymocyte precursors thymopoietin,

added to the medium, induced the appearance of antigenic markers specific for T cells (TL, Ly 1, Ly 2, Ly 3, Ly 5).

But thymopoietin was unable to restore the normal reaction to PHA 0.2 concanavalin A of lymphocytes from thymectomized mice [574].

Serum Thymic Factor (Bach and co-workers)

The compound was isolated from blood serum by two dialyses (active fraction is dialyzed in the first step and retained in the second) the successive chromatographies on Sephadex and carboxymethyl cellulose, thin-layer chromatography, and high-voltage electrophoresis were carried out [32]. The composition of the serum thymic factor was determined [129, 417]. (For an extensive review see [27]).

Bioassay of this compound is performed by the azathioprine test. Azathioprine, added to the medium, killed few of the spontaneous rosette-forming cells from mouse bone marrow but killed significantly more rosette-forming cells from bone-marrow cells preincubated with thymosin [30]. This test was further developed [33].

According to the following descriptions, azathioprine was added to the medium:

At 1 μg/ml it totally suppresses rosette-forming cells from the thymus and up to 20% from the spleen.
At 50 μg/ml it suppresses 70% from the lymph nodes.
At 100 μg/ml it suppresses up to 20% only from the spleen of thymectomized mice.

Mice were thymectomized or injected with anti-Thy 1 antiserum. The rosette-forming cells did not decrease in number. However, the azathioprine-sensitive cells decreased and the azathioprine-resistant cells increased. Incubation of these cells with thymosin or normal mouse serum resulted in an increase in the number of azathioprine-sensitive cells. Serum from thymectomized or from nude mice did not have this effect [26].

Thus, the highly azathioprine-sensitive, rosette-forming cells were presumed to be of thymic origin. Spontaneous rosette-forming cells from bone marrow are insensitive to anti-Thy 1 antiserum. Preincubation with normal mouse serum resulted in the appearance of spontaneous rosette-forming cells sensitive to anti-Thy 1 antiserum in bone-marrow cells from normal, thymeectomized, or nude mice. Nude mouse serum did not have this influence. This potency of normal mouse serum decreased by half within 150 min following thymectomy, remained constant at this decreased level for 1 week, and began slowly decreasing again later on [28]. The serum thymic factor was found in human serum. Its level is high in children and decreases with age. It decreased to zero in a patient thymectomized for myasthenia gravis [33].

The serum thymic factor is prepared from pig serum (the serum thymic factor content decreased in the serum following thymectomy in pigs [309]. Serum thymic factor, added to the medium, increased the resistance of thymocytes against cortisol (circumstantial evidence of their maturation). It induced the appearance of specific T-cell antigenic markers and an increase in cyclic AMP content in these cells [21]. In normal mice, one single injection of serum thymic factor increased about 5–8 times the number of rosette-forming spleen cells [561].

The validity of induction of antigenic markers, characteristic of T cells, as a test for thymic hormone action cells was questioned. Thus differentiated, they did not necessarily show T-cell properties in other tests, such as helper function or graft-vs-host reaction. Only thymopoietin, thymosine, and thymic humoral factor were found to be able to induce in vitro a total T-cell maturation [479]. Furthermore, serum thymic factor injections in thymectomized mice did not restore the normal reaction of their lymphocytes to phytohemagglutinin and to concanavalin A [574].

A demonstration of thymic origin of the serum thymic factor was attempted [33]. Grafts of thymic epithelium (thymomas or thymic grafts borne 8 days previously by a first recipient, in which thymocytes are known to be dead), enclosed in millipore chambers, were implanted into neonatally thymectomized mice. In the spleen of these mice the azathioprine-sensitive, rosette-forming cells increased in number. This proves that thymic epithelium exerts its influence through a millipore membrane. Nevertheless, some doubts arose as to whether serum thymic factors are secreted by the thymus. A thymic extract was prepared from calf thymus using *Bach*'s method. This extract did not induce the development of T-cell rosettes when injected into nude mice. On the other hand, serum thymic factor activity was found in similar calf thymus extracts when this factor was added to the thymus homogenate before the extraction. Thus, the extraction did not destroy the serum thymic factor presumably contained in the thymus [284]. This observation rejects by no means the idea that the thymus is involved in the production of serum thymic factor. Its site of production remains, however, to be found.

Lymphocyte Stimulating Hormone (Luckey and co-workers)

A saline thymic extract was precipitated with 20% ammonium sulfate. The precipitate was dissolved in water and precipitated with methanol. The redissolved precipitate was fractionated by successive chromatography on diethylaminoethyl cellulose and Sephadex. It was proved homogeneous by polyacrylamide electrophoresis [329, 434].

The original bioassay test for monitoring the extraction and purification is based essentially on the acceleration of the development of the lympho nodes in newborn mice [351]. The influence of this compound

on the immune response was demonstrated. Injected into new-born mice, the fraction known to induce the accelerated lymph node maturation induced also the increase in the number of plaque-forming cells against sheep erythrocytes in the spleen of unimmunized newborn mice (background plaque-forming cells) and of newborn mice injected with sheep erythrocytes [91, 247, 434]. From the impurities discarded by electrophoresis, a fraction was identified which antagonized the lymphocyte-stimulating factor.

Lymphocyte-stimulating activity was found in calf spleen extracts prepared and fractionated by *Luckey*'s method (about six times less than that from calf thymus). This may be correlated with the observations of *Comsa* and co-workers [124, 121] who found a roughly similar relation in the rat thymus and spleen content of homoeostatic thymic hormone.

Thymus Polypeptide Fraction (Milcu and Potop)

The preparation is described in [334]. It is a crude extract, and although not tested with the survival test, it was proved active in thymectomized rats with an impressive number of metabolic tests [356]. Its influence on the immune response was demonstrated by the observation of an increased antibody production in hamsters injected with influenza virus and in X-irradiated rabbits injected with HO *Salmonella* antigen. As far as we know, the influence on immune response has not been tested in thymectomized animals.

This extract was shown to delay the development of malignant tumors induced by carcinogens (such as methylcholanthrene or dimethylaminoazobenzene) or grafts (Guerin's tumor) in rats. This was also observed in cultures from a cancer derived from buccal epitheliums (KB tumor) [356]. It was also alluded to above that there is a delayed influence of the thymus on cancer development and an acceleration of cancer development in thymectomized animals. This has generally been interpreted as a thymic influence on cancer immunity. The observations made on cancer cultures hardly fit into this hypothesis.

1.10.4.3 Lipidic Extracts

The first attempt to prevent the consequences of thymectomy in guinea pigs with a lipidic thymic extract was mede by *Bomskov* and co-workers [see 113]. It was a total failure.

Isakovic and co-workers [270] extracted calf thymus with chloroform and methanol. The extract, suspended in saline, was injected into neonatally thymectomized rats. This treatment prevented the delay in growth and the decrease in lymphocyte count following thymectomy. Rats, thus treated, were immunized against bovine serum albumin at 60 days, and the delayed hypersensitivity test was performed 18 days later. The reaction

decreased in untreated thymectomized rats and was normal in those injected with the extract.

The Thymosterines (Potop and Milcu)
Calf thymus was extracted with acetone and the residue was extracted with ether. Phospholipids were discarded by cold acetone from the dried etheric extract. The residue was fractionated by successive chromatographies [see 421 for details].

First fractionation separated two fractions: III B stimulating and II B inhibiting tumor development. By several chromatographies of II B a steroidic fraction was obtained (fraction S) strongly inhibiting tumor development. This fraction was named Thymosterin. Fraction S inhibited in vitro the development of KB tumor and human thyroid cancer cultures. In vivo, it delayed the growth of carcinogen-induced and grafted tumors, such as Jensen's, Walker's, and Ehrlich's sarcoma and Guérin's adenocarcinoma. Tumor grafts in thymosterin-injected rats showed signs of regression, such as necrosis and fibrosis. Walker sarcoma grafts decreased in size in thymosterin-injected rats, and these rats survived without exception when all untreated tumor-graft rats were dead.

The influence of this extract on immune response was tested on mice injected with sheep erythrocytes. Hemolysin titers and plaque-forming cell development were significantly increased in mice injected with the partially purified fraction II B. Thus, the influence of thymosterin on tumor development in vivo can be understood as expressing the stimulation of immune functions. The influence of thymosterin on tumor development in vitro cannot. The immunostimulant action and the retardant action on tumor cultures are certainly two different phenomena. Since the immunostimulant effect was demonstrated with the partially purified fraction B (and, as far as we know, not with the presumably pure thymosterin), it is open to discussion whehter these two actions express the activity of the same chemical entity.

2 The Adrenal Cortex

Adrenalectomized and normal rabbits were injected with typhoid vaccine. The adrenalectomized animals produced three times more antibodies [267]. Normal and adrenalectomized rats were injected with sheep erythrocytes. Lymphocytes from their lymph nodes and spleen were incubated and the hemolysin released into the medium was determined. It was lower in adrenalectomized rats [433]. Thus from the very start the observation appeared controversial.

Adrenalectomy was said either to result in an accelerated rejection of skin allografts in mice [e.g. 90, 319, 353] or to show no influence [48, 351]. Still, these observations raise some doubt. The adrenalectomized mice were "protected" from the consequences of adrenalectomy on electrolyte metabolism by repeated injections of desoxycorticosterone in large amounts (1–2.5 mg once a week, as microcrystalline suspensions). This is a very important source of error. Strictly speaking, the authors never saw a truly adrenoprivic mouse. Allograft rejection was slightly accelerated in adrenalectomized mice protected just by NaCl added to the drinking water [89, 90, 238].

In Sprague-Dawley rats grafted with DA rat skin (a H 1 incompatibility) adrenalectomy resulted in a slight delay in skin allograft rejection from 7.1 ± 1.3 days in normal rats to 12.2 ± 1.5 days in adrenalectomized animals. Additional thymectomy was followed by an additional delay (to 15.9 ± 1.6 days). In Sprague-Dawley rats grafted with DB rat skin, adrenalectomy had no influence on the graft rejection [48].

Most of the experiments on the influence of the adrenal cortex on the immune response were performed on normal animals injected with large doses of corticosteroids (see [576] for a recent review).

The earliest experiments, performed with adrenocortical extracts of an unknown composition, showed that repeated injections of these extracts, together with, before or immediately after the injection of an antigen into normal rabbits [96, 195, 18] enhanced antibody production. This was explained as a result of the well-known deleterious influence of adrenocortical hormones on lymphocytes [147]. The increased amounts of antibodies in the serum were supposed to be a result of their release from the lysed lymphocytes. This explanation is not exhaustive. The lysis of lymphocytes takes place within hours after corticoid injection, whereas the increase in the antibody level in the serum is gradual, occurring over several weeks [96]. In addition, the mature, differentiated lymphocytes, participating in the immune response, are not lysed by corticoids [e.g. 106, 107]. Cortisone or cortisol injections in milligram amounts, before or together with the antigen but not afterward [166], were shown to result in a decreased production of antibodies [e.g. 52, 53, 61, 143, 153, 179, 190, 200, 203, 250, 253, 335, 341, 352, 373].

In mice, injection of 2.5 mg cortisol resulted in a decrease of in vitro spontaneous killer activity of their spleen cells [257]. In mice, IgM and IgG hemolysin production is totally suppressed when cortisone is given 4 days previous to the antigen (sheep erythrocytes). Administered after the antigen, cortisone injection results in suppression of IgG antibodies only [168]. In previously immunized rabbits, a single cortisone injection suppressed the secondary response [191]. Sheep erythrocytes are lysed with a significant delay by macrophages of rabbits injected with cortisone

[280]. In frogs, cortisone injections were followed by an increased antibody production [60].

In all these experiments cortisone or cortisol was administred in large, arbitratily chosen, doses. A quantitative relation between the injected corticoid and the injected antigen dose could be roughly demonstrated: in rats, cortisone injections (4 mg) suppressed the anti-sheep-erythrocyte antibody production. This could be restored if the antigen dose was increased a 1000-fold [54]. In mice, the production of hemolytic antibodies against sheep erythrocytes was totally suppressed by an injection of 5 mg corticosterone, whereas 1.0 mg or 2.5 mg were followed by a partial suppresion only. Desoxycorticosterone was found to be inactive [389]. In rats, single injections of 5 mg desoxycorticosterone were followed by an enhanced antibody production [97].

Allograft rejection was delayed by cortisone injections in rabbits [58], guinea pigs [476], and rhesus monkeys [305]. Desoxycorticosterone was inactive [305]. Aldosterone injections also delayed the allograft rejection in rats but so did spironolactone [48]. Metopyrone inhibited the in vivo graft-vs-host reaction [1]. Thus, corticoids and corticoid antagonists showed similar effects. The development of grafted [5, 374] or virus-induced [345] tumors was accelerated by cortisone injections.

In all these experiments, steroids were administered in milligram amounts to intact animals. These observations were not entirely confirmed in doses closer to normal conditions. Adrenalectomized or adrenalectomized and thymectomized Sprague-Dawley rats were grafted with DA rat skin. They were implanted with pellets of aldosterone, corticosterone, desoxycorticosterone or cortisol, alone or in combination. They resorbed daily 1 μg of aldosterone or 130 μg of the other steroids. Those are mounts just sufficient to ensure survival. In these animals:

Adrenalectomy was followed by a slight delay in the allograft rejection (from 7.1 ± 1.3 to 12.2 ± 1.6 days).

Additional thymectomy was followed by an additional delay (15.8 ± 1.3 days).

Implants of aldosterone, corticosterone or desoxycorticosterone partially suppressed this delay (8.5–10.0 days) in both adrenalectomized and adreno-thymectomized animals.

Implants of two steroids reestablished at least the normal rejection period (6.7–7.0 days). A third implant had no additional influence.

Cortisol implants were by themselves inactive, but additional cortisol implants delayed the rejections in animals implanted with one or several of the other steroids [120].

There was no difference between the corticoid effect in adrenalectomized and adreno-thymectomized rats. Furthermore, in adreno-thym-

ectomized rats injected daily with the *Bernardi-Comsa* thymic hormone preparations, this additional treatment had no influence on the allograft rejection [117]. As soon as corticoids were administered, the influence of the thymus disappeared.

From this experiment we can identify under physiological conditions: (a) three immunostimulant corticosteroids (aldosterone, corticosterone, and desoxycorticosterone) and (b) a minor immunosuppressive steroid, cortisol (this is not exactly under physiological conditions, since rats do not produce cortisol).

This conclusion is oversimplified. Under adequate conditions all corticosteroids known thus far have shown an immunostimulant effect.

Rabbits were immunized against bovine serum albumin or diphtheria toxoid, and 40–100 days later their lymphocytes were incubated with sheep erythrocytes coated with the same antigens. These erythrocytes were lyzed if the medium contained at least 1 ng/ml and less than 0.1 μg/ml cortisol [10]. Without added cortisol, the hemolysis occurred only if the incubation medium contained 25% fetal calf serum.In higher concentrations cortisol added to the medium suppressed antibody production in vitro [109], [e.g. 380], cortisone (70 μg/ml was ineffective on the in vitro antibody production of immunized lymphocytes [179]. It should be noted that metopyrone injections inhibited the graft-vs-host reaction in rats [1]. In conclusion, the dose-response curves of corticoids on the immune response appear to be bell shaped.

Cortisol acts as an immunostimulant at 1 ng/ml of medium and as an immunosuppressive at 0.1 μg/ml [10].

Aldosterone acts as an immunostimulant at 1 μg/day [117], as a partially immunosuppressive at 1 mg/day, and as a totally immunosuppressive at 5 mg/day [389].

Desoxycorticosterone still acts as an immunostimulant at 5 mg/day [97].

The immunosuppressive dose range is unknown. Perhaps it exists since desoxycorticosterone was prophlogistic at milligram doses and antiphlogistic at 25 mg/day [432]. This is, of course, a rough estimate. The results may differ within enormous limits according to the test used. For instance, cortisol was found to be immunostimulant on incubated human B cells when added in such enormous concentrations as 10^5 [567].

Stress was generally followed by a depression of the immune response. This was obtained by overcrowding [473, 518], by training to avoid electroshock announced by a light-signal [473, 547], by adaptation to high altitues [77], by ether anesthesia [207] or by short exposure to a temperature of 37°C [416]. In fact, the observations are not easy to interpret. During the general adaptation syndrome, distinguishable stages should be considered: (a) the alarm stage, lasting several hours, and characterized

by a relative adrenocortical insufficiency; (b) the resistance stage, lasting for weeks, characterized by an enhanced corticoid production and by symptoms of hyperadrenocorticism and (c) the terminal exhaustion stage, characterized by adrenal deficiency. Thus, the effects of stress are certainly different in terms of duration. Examination of the adrenals is a necessary complement to those experiments. It was performed by *Gisler* [207] who found an approximately twofold increase in the plasma corticosterone level in mice following ether anesthesia. A detailed dose-response curve of corticosterone on immune response would be useful. The experiments quoted above simply indicate the possibility of plotting this curve. It is generally believed that corticosteroids inhibit the immune response inasmuch as they kill cells [e.g. 147, 150]. Which cells are killed? Adrenalectomy is followed by thymic hypertrophy, but the hormone content of this big thymus decreases [124, 137].

Administered in large amounts, cortisol induces a wave of pyknoses in the thymus and to a lesser extent in the lymph nodes [see 147]. Small lymphocytes were primarily killed by this method [182, 151]. Added to the medium, cortisol (0.01 μg/ml) or corticosterone (0.1 μg/ml) shortened the survival time of lymphocytes [291, 462, 519]. At 2.0 μg/ml cortisol killed the small lymphocytes from embryonic mouse thymus. At 50 μg/ml it killed all cells [430]. The surviving cells show an increased helper potency [106] and an increased potency to induce the graft-vs-host reaction [65, 107]. Following a single injection of 2 mg of cortisol the spontaneous Thy 1 positive, rosette-forming cells did not decrease in number, while total lymphocyte count decreased by 90% [33]. In conclusion, cortisol killed the immature cells only. This observation was confirmed over and over again.

Cortisol appeared to kill other cells, besides the small (immature) thymic lymphocytes. In mice injected with 15 mg of cortisol, the mononuclear cells disappeared from the blood [497]. Three hundred micrograms of cortisone/g body wt. were injected into mice. Spleen cells from these mice did not differentiate into cytotoxic cells when incubated with allogenic spleen cells, however, they developed helper cell function. It was concluded that another member of the cell-to-cell interaction was killed [316]. Speculatively this cell was identified as the macrophage. Spleen cells from mice, stressed by ether anesthesia, produced few plaque-forming cells with sheep erythrocytes. Addition of syngeneic T cells did not result in any increase in their number, whereas a significant increase in plaque-forming cells was observed following the addition of syngeneic B cells and macrophages. In conclusion, stress results in damaging B cells and macrophages, but not T cells [207]. It remains open to discussion as to how stress resulted in this effect. It should be noted that at weak concentrations (10^{-8}) cortisol induced an increased proliferative activity

in incubated lymphocytes [430, 539]. Dose response curves of these corticoid influences are much needed. From these observations, it is not possible to understand the immunostimulant influences of corticoids administered in small amounts. Concerning this peculiar aspect of this phenomenon, we only know that the rejection of a second skin allograft is not influenced by adrenalectomy in rats which had already rejected a similar first graft:

	Rejection time (days)	
	First Graft	Second Graft
Normal rats	7.1 ± 1.3	4.3 ± 0.5
Adrenalectomized	12.2 ± 1.5	4.4 ± 0.5
Adrenalectomized and thymectomized	15.9 ± 1.6	4.6 ± 0.5

Thus, the immunostimulant effect of the adrenal cortex appears to express an influence of corticosteroids on the differentiation of immunocompetent cells. As soon as this occurs, corticoids are no longer necessary. The influence of corticoids and of thymic hormones seems to be exerted on the same stage of the immune response.

3 The Thyroid

To our knowledge, the first observation concerning the role of the thyroid in immune response was made by *Houssay* and *Sordelli* [260]. Thyroidectomized rabbits and horses were injected with sheep erythrocytes, typhoid vaccine or tetanus or diphteria toxoid. They produced less antitoxins than intact ones, whereas the agglutinin and hemolysin titers of their serum were equal to those of intact animals.

Rats were thyroidectomized at birth. They were injected with sheep erythrocytes at the age of 2 months. Plaque-forming cells in their spleen decreased in comparison with intact rats. Daily thyroxine injection (10 micrograms/animal, the reason for this dose is not explained) restored the normal reaction [173].

Humans were injected with sheep erythrocytes or antigens of *Shigella flexneri, Salmonella typhi* or *Escherichia coli.* The antibody production was higher in Graves' disease patients and lower in hypothyroid patients than in healthy subject [501].

Guinea pigs were inoculated with tuberculosis bacilli and injected with a lethal dose of tuberculin. In intact animals this induced a rise in body temperature, but in thyroidectomized animals it did not. Serum from tuberculosis-injected guinea pigs was injected into normal animals together with tuberculin. The temperature of recipients rose if the serum donor were intact, but not if the donor were thyroidectomized [288].

One-month-old male Sprague-Dawley rats received DA skin grafts. The recipients were thyroidectomized, thyroidectomized and thymectomized, thyroidectomized and adrenalectomized or subjected to all three operations. Thyroidectomy by itself delayed graft rejection (7.1 ± 1.1 days – 9.7 ± 1.3 days). In thymectomized, adrenalectomized and thymo-adrenalectomized rats the rejection (delayed already by these operations) was not further delayed by thyroidectomy. Daily thyroxine injections (5 μg/100 g, the dosage known to be sufficient to prevent thiouracil goiter) restored the normal rejection in thyroidectomized rats, but had no influence on thymectomized, adrenalectomized or thymo-adrenalectomized [118]. Thus, at least in the peculiar case of graft immunity:

1. The immune response is depressed by thyroidectomy and restored to normal by thyroxine in thyroidectomized animals.

2. Neither thyroidectomy nor thyroxine influences the immune response in the absence of the thymus or the adrenals.

The influence of the thyroid on the immune response appears to be mediated by the thymus and the adrenal. (Both thymus and adrenal are atrophic following thyroidectomy, see Sect. 6.2.2–6.2.4.)

4 The Gonads

Female mice are better humoral immune responders than males [e.g. 46, 215, 477]. F (C 57b1 × BALB) mice were injected with erythrocytes from BPS mice. Their serum was fractionated in order to separate 7S and 19S antibodies. In males hemagglutinins were present in the 19S fraction only, whereas in females both fractions contained hemagglutinin [45].

Skin allograft rejection was faster in females than in males [90, 238, 472]. Castration was followed by an accelerated allograft rejection in males, less so in females [90, 238, 472]. Syngeneic grafts of ovaries in males and of testes in females had no significant influence on allograft rejection [90, 238, 472]. Testosterone injections (650 μg three times weekly) delayed the rejection in castrates [89]. In rats similar results were observed [119], the production of hemagglutinins against allogeneic mouse erythrocytes increased in castrates but hemagglutinin production against sheep erythrocytes decreased. Estradiol injections in male mice were followed by an increased antibody production (e.g. 2.5 μg daily [287], 10 μg [477]). Allograft rejection was delayed following estradiol injection (50 μg three times weekly) in X-irradiated mice reconstituted with syngeneic bone marrow [496]. The development of adjuvant arthritis (by injection of Freund's adjuvant) or autoimmune thyroiditis (by injection of thyreoglobulin) was delayed in female rats by daily injections

of 0.5 mg estradiol and in males by daily injections of 0.5 mg testosterone [279]. In F (C 57b1 × BALB) mice injected with BP8 erythrocytes, hemagglutinin production was increased in castrates and was depressed in castrates bearing estradiol implants (the daily resorption rate was not determined [45]). Skin allograft rejection was delayed by estradiol injection (500 μg daily) in rabbits bearing a corneal graft from the skin donor strain [583]. Thus, the enhancement of immune response by estradiol was not generally confirmend.

Thymectomized male rats were injected with estradiol (200 μg daily for 16 days). Their serum was added to the incubation medium of human lymphocytes. These cells produced less rosettes with sheep erythrocytes than those incubated with normal rat serum [578].

Castrated Sprague-Dawley rats (males and females) rejected DA skin allografts in 5–6 days (intact animals in 7.1 ± 1.0 days). Implants of estradiol in females (daily resorption rate 0.4 μg) and of testosterone in males (daily resorption rate 40 μg) restored the normal rejection. In thymectomized castrates of both sexes, the grafts were rejected as in thymectomized rats (9.8 ± 1.6 days) and sex hormone implants were of no influence. In thymectomized castrates daily injections of the *Bernardi-Comsa* thymic hormone preparation (100 μg) was followed by rejection within normal time limits, whereas the same injections were of no influence on those castrated alone. Thus, in this experiment the influence of the gonads on allograft rejection appeared to express the influence of the gonads on the thymus [119]. Receptors for estradiol were demonstrated on the surface [577] and in the cytosol [568] of thymocytes.

Female thymectomized CBA mice were mated with T_6T_6 males. They rejected T_6T_6 skin grafts in normal time limits. This is not due to sex hormones. Pseudopregnancy had no similar effect. The extirpation of the fetuses on the 10th day of pregnancy (placenta left in place) resulted in a significant delay in the graft rejection which can only be explained by the passage of hormones from the fetus to the mother [and not by passage of cells from the F (CBA × T_6T_6) fetus] [394]. In female nude mice mated with CBA males the pregnancy did not enhance the immune response against sheep erythrocytes [570].

Progesterone injections had no effect on skin allograft rejection in normal mice and rabbits [e.g. 351]. In spayed female rats bearing implants of progesterone (they resorbed 400 μg daily of the steroid), skin allografts were rejected in 9.3 ± 0.9 days, whereas spayed controls did so in 5.8 ± 1.0 days. In spayed females bearing similar progesterone implants associated with estradiol implants (they resorbed daily 0.4 μg of the latter), skin allografts were rejected within 7.6 ± 1.1 days. In other words, these rats reacted like normal females (rejection within 7.1 ± 0.8 days) and not like castrates. Estradiol + progesterone = 1:1000 is the well-known

ratio of these hormones which induces a pseudopregnancy in spayed females. Administration of estradiol and progesterone under these conditions was thus observed to suppress the consequences of ovariectomy on the immune response as well [119]. Spleen cells of pregnant mice showed a decreased activity in mixed lymphocyte culture but an increased plaque-forming cell activity. These modifications could be reproduced with injections of human chorionic gonadotropin but not of progesterone.

Osoba observed that [394] (see above):

1. Pregnant thymectomized mice reject skin grafts like normal animals.
2. Extirpation of the fetuses but not of the placentas results in a significant delay in rejection.
3. Pseudopregnancy has no similar consequences.

These observations seem to exclude the influence of the corpus luteum and to emphasize the influence of the placenta on the immune response. An immunostimulant influence seems to emanate from the fetus (perhaps from its thymus). These observations allude to the controversial problem of the mother-fetus tolerance. Mating between animals histoincompatible to each other results in a normal pregnancy and the offspring are reared normally. Immunosuppressive influences seem, therefore, to be active during pregnancy. Experimental analysis of this phenomenon is scarce. The attempts to reproduce the influence of pregnancy with hormone injections were not always performed under valid conditions, that is:

In castrated animals

Parallel experiments with injections of progesterone alone and with progesterone associated with estradiol in the well-known ratio of 1000:1, which induces the pseudopregnancy reaction in the gonaducts of ovarectomized animals.

According to *Osoba* an immunostimulant influence seems to emanate from the fetus itself and the placenta seems to exert an inhibitory influence. In fact, the role of the placenta is a matter for future research. The placenta is a plurivalent endocrine gland. The list of the hormones produced by the placenta is not yet complete, but an immunosuppressive influence may emanate from estriol, which the placenta produces in large amounts.

5 The Adenohypophysis

The description of the consequences of hypophysectomy on the immune response are divergent from one author to another. Hypophysectomized mice were injected with sheep erythrocytes but few plaque-forming cells

developed in their spleen. The normal reaction was restored by injections of growth hormone or corticotropin [209, 210]. Plaque-forming cells and serum agglutinin titers were significantly low in hypophysectomized rats injected with sheep erythrocytes [397]. Hypophysectomized Sprague-Dawley rats grafted with DA rat skin rejected those grafts with a significant delay [123]. Hypophysectomized Wistar-Furth rats produced fewer antibodies against sheep erythrocytes and rejected skin allografts later than normal rats [575]. Divergent observations were registered too. Hypophysectomized rats reacted normally to sheep erythrocyte injections [159, 277]. We cannot explain these divergencies.

The consequences of hypophysectomy on immune response were revealed only after a subsequent X-irradiation [502]. In these animals, both skin allograft rejection and hemagglutinin production after sheep erythrocyte injection were significantly depressed [277].

The observations on immune response in Snell-Bagg dwarf mice were explained. These mice are dwarves inasmuch as they suffer from a severe adenohypophyseal deficiency (see Sect. 6.2.2). Dwarf mice are severely immunodeficient. Following sheep erythrocyte injection they develop significantly few plaque-forming cells in the spleen [36, 37, 277]. Serum agglutinin titers were low after sheep erythrocyte or *Brucella* injections [157]. Dwarf mice did not reject allografts [175]. The secondary immune response of dwarf mice to sheep erythrocytes was normal [38]. Nevertheless, thymocytes or spleen cells from dwarf mice induced the in vivo graft-vs-host reaction if injected into F (SB × AKR) mice [158].

6 Influence of Hypophyseal Hormones on the Immune Response

6.1 Growth Hormone

Growth hormone injections in hypophysectomized mice were followed by restoration of the immune response [209, 210]. Growth hormone injection in dwarf mice was followed by activation of the immune response [e.g. 175, 397]. Thyreotropin and thyroxine had a similar effect [37]. The enhancement of the immune response (number of plaque-forming cells in the spleen after injection of sheep erythrocytes) was particularly significant when growth hormone and thyroxine were injected together [413]. The thymus is involved in this effect of growth hormone. In thymectomized dwarf mice, growth hormone injections had no effect on the immune response, but they induced normal growth [174]. This involvement of the thymus in the influence of the growth hormone on the immune response was observed in hypophysectomized rats. In hypophys-

ectomized and thymectomized rats the dose-response ratio of growth hormone to growth was low although some effect was observed [see 329 for review]. However, the growth hormone injections had no effect on the immune response of these rats. Hypophysectomized and thymectomized rats produced no antibodies against human serum albumin. The normal response was restored by injections of growth hormone and the *Bernardi-Comsa* thymic preparation, not by growth hormone or thymic hormone alone [125]. Skin allograft rejection was delayed to the same extent in hypophysectomized and in hypophysectomized thymectomized rats. In those only hypophysectomized, the normal reaction was restored by growth hormone injections; in those thymectomized and hypophysectomized, growth hormone and thymic hormone injections had the same effect (but not growth hormone or thymic hormone alone [123]). These observations may explain the influence of thyreotropin or thyroxine on the immune response of dwarf mice. Thyroxine is a permissive factor for the secretion of thymic hormone [124] and indeed thyroxine injections enhanced the immune response in dwarf mice, but not in neonatally thymectomized mice [16] of normal strains.

6.2 Corticotropin

The humoral immune response was depressed by corticotropin injections. Antibody production against ovalbumin [190], pneumococcal antigen [203, 62], horse serum [250], typhoid vaccine [373], and bovine serum albumin [341, 389] was severely inhibited following corticotropin injection in large doses. There were remarkably few plaque-forming cells in the spleen of intact mice when these were injected with corticotropin and sheep erythrocytes.

On the allograft immune response corticotropin influence was less obvious. A slight delay was observed in mice [351] and no influence was observed on rhesus monkeys [305], rabbits [9], guinea pigs, pigs or humans [170]. These experiments were carried out on intact animals. The corticotropin dosage is difficult to understand, since corticotropin doses were expressed in units of weight from the start, regardless of the successive purifications.

In hypophysectomized and thymectomized rats, daily injections of 300 milliunits of corticotropin were followed by a remarkably accelerated skin allograft rejection (17.8 ± 1.2 days – 6.7 ± 0.8 days). Additional injections of the *Bernardi-Comsa* thymic hormone preparation resulted in a nearly complete suppression of the corticotropin effect (rejection in 15.2 ± 1.2 days) [123]. From previous experiments it was known that thymic hormone is a synergist to growth hormone and that it antagonizes

corticotropin. The immunological tests mentioned above gave results consistent with this notion.

There is little to be said about the influence of other adenohypophyseal hormones on the immune response. The restoration of a normal response to sheep erythrocytes with thyreotropin injections [175] has been mentioned already. In hypophysectomized and thymectomized rats thyreotropin injections had no influence on skin allograft rejection (*J. Comsa* and *H. Leonhardt,* unpublished observations). Thus, the influence of thyreotropin may be mediated by the thymus via the thyroid, see Sect. 3.

Pancreatectomy was followed in rats by a delayed rejection of skin allografts and no change in the immune response to sheep erythrocytes, *Brucella* antigen or bovine serum albumin. As far as we know, this is the first attempt to investigate the influence of the pancreas on immune functions.

7 Mechanisms of Hormonal Influence on the Immune Response

This problem can be considered from two aspects:

1. Events on the cellular of subcellular level which may enable us to understand the hormonal influences.
2. All the hormones mentioned above are present in normal animals. Intricate interactions between them were demonstrated by several tests. To what extent could these interactions influence the immune response? Can we imagine a hormonal coordinating mechanism for immune functions?

7.1 Cellular and Subcellular Aspects of Hormonal Action

The following summary is by far inexhaustive. This would unreasonably exceed the limits of this review.

7.1.1 The Corticosteroids

Corticoids enter the cells, are fixed to a transcortin analog in the cytosol [e.g. 322, 383, 497; see 187], traverse the cell thus bound, and are finally fixed to specific receptors in the nucleus [e.g. 449, 466, 546]. This is completed within minutes [466]. The nuclear receptor was thought to be a histone [467] or an acidic protein [e.g. 2]. The binding protein is perhaps essential for corticoid action within the nucleus. Purified "chromatin" is able to induce RNA synthesis in vitro. This synthesis is depressed by protein-bound cortisol, but not by free steroids [446]. The presence

of specific corticoid receptors on lymphocytes is essential for the cytotoxic corticoid effect. Incubated with cortisol, the corticoid receptors of lymphocytes decrease with time in the surviving cells [49]. (The cells bearing a dense receptor net are killed.)

Corticoids increase the stability of cellular and subcellular membranes. Hemolysis by antibodies or by physiochemical agents is inhibited by corticosteroids [e.g. 4, 272]. Hamster kidney cells infected with rubella virus are not lysed by antirubella serum if cortisol is added to the medium [498]. Intracellular membranes were also found to be stabilized by adding cortisol [162]. Cortisol inhibited the release of lysosomal enzymes into the cytosol [532, 534]. Corticoids interfere with glucose metabolism. Glucose transport and phosphorylation [69, 383], glucose uptake [281, 375], and ATP synthesis [391] are inhibited by adding cortisol (at 10^{-7} – 10^{-6} M concentration). Metabolism by the pentose cycle was found to be enhanced [69], this action is blocked, however, by puromycin [376, 339]. It was concluded that cortisol induces the synthesis of a proteidic inhibitor of glucose metabolism.

Corticoids inhibit DNA and RNA synthesis [see 495 for review]. Thymidine uptake [339, 340, 376], DNA polymerase [290], and thymidine-kinase activity [241] are depressed, also uridine uptake [199, 551], adenine uptake [77], RNA polymerase activity [2, 68, 194, 340], and DNAse activitiy is enhanced [443].

Corticoids inhibit protein synthesis in most tissues (except in the liver). Amino acid uptake [e.g. 64, 181, 227, 375, 537] is depressed in presence of cortisol and so is the activity of various transaminases [e.g. 64]. The inhibition of lymphokine synthesis [523] is particularly relevant to our topic. Amino acid release by incubated lymphocytes is enhanced in presence of cortisol [489]. Thus, cortisol appears to inhibit the anabolism and to promote the catabolism of nucleic acids and proteins. These effects were obtained with cortisol at high concentrations (10^{-7} – 10^{-6} M). They are supposed to be direct with no second messenger needed [495]. Yet cortisol injections depressed the adenylate-cyclase activity in X-irradiated rats [285].

It is interesting to note that the stimulating effect of cortisol at low concentrations on lymphocyte proliferation in vitro (10^{-8} M [539]) was supposed to be mediated by cyclic AMP. It should also be noted that the stabilizing effect on lysosome membranes in vitro was observed at low, but still immunostimulant concentrations [144]. These two observations are the only ones possibly related to the immunostimulant influence of corticoids within the physiological range.

7.1.2 Peptidic Hormones

It is generally accepted that peptidic hormones do not enter the cell. Thus a "second messenger", transporting the hormonal message from the cell membrane (to the nucleus?), becomes relevant.

7.1.3 Cyclic Adenosine Monophosphate (cAMP)

Added to the medium, cyclic AMP stimulates lymphocyte proliferation [333]. Injection of sheep erythrocytes into immunized mice resulted in an increase in the cyclic AMP level in their spleen lymphocytes within 2–10 min [418]. When (butyryl)- cyclic AMP was added to the medium [e.g. 70, 266, 530], plaque-forming cells increased in number in spleen cells from mice immunized against sheep erythrocytes. Adenylcyclase stimulating catecholamines (e.g., epinephrine) enhanced the immune response [70] and so did those drugs which inhibit phosphodiesterase [72]. This is, however, only valid up to a certain point. When added in excessive amounts, cyclic AMP inhibited plaque-forming cell differentiation [73]. The cytolytic activity of immunized C57 b1 mouse spleen cells against DBA mastocytoma was inhibited by cyclid AMP [254]. Plaque-forming cell formation was inhibited in mouse spleen cells by cyclic AMP added to the medium. (Was it added in excess in these two experiments?) Rosette-forming cells increased in number in human lymphocytes by administration of drugs which increase the cyclic MP content of lymphocytes and then decreased when dibutyryl-cyclic AMP was added to the medium [99] (perhaps in excess, see above).

7.1.4 Synthetic Polyribonucleotides

The enhancing effect of poly (A:U) on the immune response [56, 72, 74, 73, 266, 343] at low concentrations (high concentrations show an opposite effect [72]) was interpreted as an enhancing influence on cyclic AMP synthesis [70]. Poly (A:U) injected into neonatally thymectomized mice induces a normal rosette-forming cell differentiation and a normal allograft rejection [126]. Still poly (A:U) added to the medium enhanced cytotoxic activity of spleen cells cultivated with allogenic cells in normal mice but not in the nude ones (the thymus was necessary [56]). Prostaglandins were also said to enhance the immune response [265] and the prostaglandin synthesis inhibitors did as well [531]. In this new field the observations are still confusing.

Poly (A:U), prostaglandin E_2 or cyclic AMP, added to the medium, induced the potency of graft-vs-host reaction in spleen cells from thymectomized mice; incubation of mouse lymphocytes with thymic humoral factor resulted in an increased cyclic AMP content [299]. Incubation of

spleen cells from nude mice with thymopoietin, cyclic AMP or poly (A:U) induced the appearance of antigenic markers and helper function [451]. Neither thymosin [20] nor serum thymic factor [20] induced an increased cyclic AMP content in thymocytes when added to the medium. Also, incubation of thymocytes with serum from normal or thymectomized pigs induced an increased cyclic AMP content in these cells, whereas the serum thymic factor produced by synthesis did not [34].

Perhaps, this partly confusing picture could be somewhat clarified if we knew the consequences of thymectomy on the cyclic AMP level of lymphocytes. Remarkable as it may sound, we do not.

7.2 Hormonal Interactions

The hormones mentioned thus far are simultaneously present in the organism. The hormonal influence on immune response (i.e., its action directly on the immune system or mediated by another endocrine) is expressed by an algebraic sum. This assertion is self-evident. Thus far, the documentation concerning this topic is poor. Elements of the hormonal interactions in general must be noted. (Observations mentioned without references are described in extenso [114]). Hormonal interactions must be seen from two different aspects:

1. The reciprocal influence between gland A and gland B
2. The interaction between the hormones of gland A and gland B in their circulating form

These two aspects probably are linked to each other in some way. However, we can only assume this up to now.

7.2.1 Consequences of Thymectomy on the Endocrines

Following thymectomy, a transitory stimulation of the thyroid, and the gonads, and the adrenal cortex was observed in guinea pigs and rats [114]. These endocrines returned to normal in rats and in those guinea pigs which recovered from thymectomy. In those which waste (examined sub finem) degenerative changes were observed. This was the case in thymectomized mice. As far as we know, the endocrines were examined in wasted mice only [155]. This is insufficient. Degenerative changes in the endocrines occur in wasted animals, whatever the reason for wasting may be (e.g., in the terminal stage of deficiency diseases). The consequences of thymectomy on the adrenal cortex seem ambiguous at first sight. Evidence of stimulation was noted [see 114, 140]. However, the corticosteroid content of the systemic blood was found to be decreased [137, 177] whereas aldosterone content was increased [177]. Still, the corticosterone

level increased in the efferent blood of the adrenal [79, 470]. This discrepancy is believed to result from an accelerated denaturation of corticosterone in thymectomized animals, perhaps mediated by the stimulated thyroid. Indeed, thyroxine injections were followed by an accelerated reduction of ring A in steroid molecules [550]. A methodical investigation of the steroid turnover rate in thymectomized animals would be of interest.

In the adenohypophysis, thymectomy was followed by signs of stimulation of, more or less, all cells. The sequence of the events is different from one species to another. In rats [114, 137] and in mice [55], the stimulation of α cells occurs early. In guinea pigs [114], δ cells precede the α cells, which show signs of stimulation only in recovering animals. In wasted guinea pigs the adenohypophysis is completely dedifferentiated (consisting only of small chromophobes). In mice, the stimulation of α cells appears before the onset of wasting [415]. In thymectomized axenic mice, which do not waste following thymectomy, similar changes in the adenohypophyseal cells occurred [415, 475].

Further research concentrated on the α cells. Electron microscopy indicated an enhanced endoplasmic reticulum and an almost total disappearance of secretion grains [562, 475] in thymectomized mice. This evidence of an increased activity is consistent with the increase in the growth hormone level in the serum [137]. We noted that following thymectomy the efficiency of the growth hormone is decreased (by the loss of its synergist). This results in an increased growth hormone secretion by a negative feedback effect.

All these changes were prevented by thymic grafts in mice [54] or by daily injections of the *Bernardi-Comsa* thymic hormone preparation [see 113]. The increase in the serum growth hormone level following thymectomy was prevented by injections of the same compound [562].

The nude mouse is supposed to suffer from a severe thymic deficiency. But thymosin could be prepared from the rudimentary thymus of nude mice [573]. The histological picture of the endocrines shows to a certain extent that of thymectomized mice at the terminal wasting stage. The thyroid shows degenerative changes. In the adrenal at the age of 50 days, the reticular zone of the adrenal cortex is peculiarly large. At 90 days the cortex is shrunken. The growth hormone cells of the adenohypophysis show the same picture as in thymectomized mice. Blood thyroxine levels are low. Corticosterone levels temporarily increased (at 14 days) and later decreased. Sex steroids decreased. In vitro, adrenals of nude mice produce less corticosterone, more desoxycorticosterone, and as much aldosterone as those of normal mice. Addition of corticotropin to the medium results in normalization of the steroids produced [408, 409]. Thymic grafts in nude mice suppressed all degenerative changes in the

endocrines. The consequences of thymic extract injection on the endocrines of nude mice have not been investigated as far as we know.

7.2.2 Thymus-Thyroid

Following thyroidectomy, the thymus undergoes atrophy of the senile type in infant guinea pigs [see 114]. The hormone content of the thymus, the lymph nodes, and the spleen decreased in thyroidectomized rats. This could be prevented by daily injections of 5 μg of thyroxine, the minimal dose for preventing thiouracil goiter [124]. In guinea pigs, the minimum dose necessary was 2 μg daily. Increased thyroxine amounts induced thymic hypertrophy (up to 16 μg). Larger amounts, however, resulted in a relative atrophy with histological signs of intense stimulation, which points to the picture of human thymus in Graves' disease. The lymphocytosis induced by thyroxine injections is mediated by the thymus. In doses inducing lymphocytosis in normal guinea pigs, thyroxine injections are followed by lymphopenia in thymectomized animals [114].

The thyroid seems to regulate in some way the thymus functions. Thymic hypertrophy induced by castration is inhibited by simultaneous thyroidectomy [344] as well as an increase in the hormone content of the thymus in castrates [124].

The influence of the thyroid on the thymus is probably direct. Thymic hormones and thyroxine are mutual antagonists (demonstrated with the *Bernardi-Comsa* compound [see 114] and with thymosin [see 127]. The influence of the thymus on the thyroid (the thyroid stimulation following thymectomy) is more intricate. The thymus antagonizes thyreotropin and it decreases the thyreotropin secretion [see 114].

7.2.3 Thymus-Adrenal

The influence of the adrenal on the thymocytes was described above. In addition, an adrenal influence on the thymic glandular epithelium seems probable. Adrenalectomy is followed by thymic hypertrophy [see 114 for review], but the hormone content of this enlarged thymus is low [124, 137] and increased above the normal level in adrenalectomized rats with corticosterone or desoxycorticosterone [124].

The influence of the thymus on the adrenal cortex is intricate. The stimulated condition of the adrenal cortex following thymectomy (prevented by thymic hormone injections) was mentioned above. The adrenal of thymectomized rats was incubated in vitro. Addition of thymosin to the medium was followed by an increased oxygen consumption. The oxygen consumption of adrenals from thymectomized mice previously injected with thymosin or with homeostatic thymic hormone was low

(equal to that of normal mice if homeostatic thymic hormone was injected, but still 30% higher after thymosin injections [140]). Obviously, two mechanisms interplay: (a) the antagonistic action of the thymus toward corticotropin (see above) and (b) a possible direct action of the thymus on the adrenal cortex.

The thyroid seems to play a permitting role on the adrenal's influence on the thymus. The "big empty thymus" of adrenalectomized rats is not seen in tyhroidectomized and adrenalectomized rats. In these rats, the thymus shrinks just as in those thyroidectomized alone.

7.2.4 Thymus-Adenohypophysis

Following hypophysectomy the thymus shrinks, and the picture is that of senile involution. The lobules shrink, the cortex is narrow but particularly well delimited from the medulla, there are no lymphocytes in the medulla, and the lymph nodes shrink [452]. Thymidine uptake is decreased in the thymus and in the spleen [293]. The validity of this test could be questioned because hypophysectomy had no effect on thymidine uptake in the testes [293].

Antigrowth hormone antiserum induced a similar thymic atrophy [411]. The hormone content of the thymus decreased as well as the stores of thymic hormone [124, 128, 137] in the lymph nodes and the spleen. Daily injections of growth hormone, corticotropin or thyreotropin prevented these changes [124]. The shrunken lymph nodes of hypophysectomized rats are restored to normal by growth hormone injections.

These observations may demonstrate:

1. An *influence* of *growth hormone* on thymic lymphocytes, this is an acquired characteristic. There are specific receptors for growth hormone on the surface of lymphocytes [e.g. 397]. Im lymphocyte cultures growth hormone addition is followed by an increased mitosis rate [333], an increased thymidine and uridine uptake [397, 490]. This action is supposed to be mediated by cyclic AMP since addition of coffeine to the medium decreased the minimal efficient growth hormone concentration by two-thirds [333]. Growth hormone seemed to favor the maturation of thymocytes. Thymectomized mice, injected with sheep erythrocytes, develop few plaque-forming cells. Injection of growth hormone and thymocytes was followed by an increase in the plaque-forming cell number, whereas neither growth hormone alone, nor thymocytes alone had any effect [16]. Injection of newborn Long-Evans rat spleen cells in newborn Ch River rats (of Sprague-Dawley origin) induced runting syndrome only when injected together with growth hormone. The influence of growth hormone thus appears to be exerted on Stutman's postthymic immature cells.

2. An *influence* of *growth hormone* on the secretory thymic reticuloepithelium. This ir purely hypothetical.

It has also been documented that growth hormone is a synergistic to thymic hormone (see above).

A clear-cut difference appears between the stimulatory influence of growth hormone on the thymus and the synergism between growth hormone on the thymus and the synergism between growth hormone and thymic hormone.

It has been mentioned already that corticotropin injections restored the hormone content of the thymus, the lymph noces, and the spleen in hypophysectomized rats. The mechanism of this phenomenon is open to discussion. It can be understood as being mediated by the adrenal cortex (see above) but a direct influence of corticotropin on the isolated rat thymus has also been observed [137]. The restoration of the hormone content in the thymus of hypophysectomized rats by thyreotropin can certainly be understood as being mediated by the thyroid.

Injections of an antigrowth hormone antiserum in 27-day-old mice resulted in a wasting syndrome similar to that induced by neonatal thymectomy. The thymus of these animals shrunk and their immune response became deficient. In their hypophyses the growth-hormone cells were modified as in thymectomized mice. Their thymuses shrunk [475]. Injections of an antithymus antiserum had no similar effect, perhaps because it was principally an antithymocyte serum. For this test, antithymic antisera should be prepared with cells from the secretory thymic reticuloepithelial cells.

The dwarf mouse suffers from a severe adenohypophyseal deficiency [e.g. 40, 321, 471]. This deficiency is conditioned by the adenohypophysis itself and not by the hypothalamus. The graft of a syngeneic normal hypophysis in the sella turcica of dwarf mice was followed by normal growth [88]. Substitutive therapy with adenohypophyseal and other hormones to some extent produced confusing results. Dwarf mice resumed growth when injected with growth hormone, thyreotropin [47] or thyroxine [236, 390, 472]. Bioassay and polyacrylamide electrophoresis of a crude hypophyseal extract of dwarf mice demonstrated a severe growth hromone deficiency [321]. Thus, dwarf mice are dwarves inasmuch as they are deficient in growth hormone (in fact, the other hypophyseal hormones have not been researched as far as we know).

The thymus, the lymph nodes, and the splenic white pulp are atrophic in dwarf mice. At 20 days the thymus shows senile atrophy, and at 60 days the terminal stage of deficiency diseases [36]. Yet this thymus was proven functional (see Sect. 5). Thyroid and adrenals are atrophic.

The restoration of dwarf mice by thyreotropin or thyroxine injections raises some problems. The restoration of the immune response by thyroxine

may result from the influence of thyroxine on the thymus. We know, indeed, that thyreotropin restored the thymus function in hypophysectomized rats. Unfortunately, we do not know which changes in the thymus of dwarf mice are induced by thyroxine. In any case, this explanation is unsatisfactory since thymic hormone was shown to be of no influence on the immune response in hypophysectomized animals, unless growth hormone had also been injected (see above). Thus, we could suspect that thyroxine injections in dwarf mice induced an increased growth hormone secretion also. It is, indeed, open to discussion whether the adenohypophysis of dwarf mice is asleep or dead. In the first case, thyroxine may induce a chain of events in the adenohypophysis of thyroidectomized rats, the growth hormone content tending toward zero. It is restored with thyroxine injections [321]. There is circumstantial evidence that the well-known hypertrophy of the adrenal cortex following thyroxine injections is mediated by the adenohypophysis [e.g. 164]. Corticotropin injections resulted in an increased growth hormone secretion [e.g. 480, 555]. In dwarf mice growth hormone injections showed a gonadotropic effect. Puberty was induced and fertilization, pregnancy, and breeding were possible [39]. An intricate interaction within the cells of the adenohypophysis could be possible in the particular case of the dwarf mouse which is injected with one or another hypophyseal hormone. This presumption indicates that the adenohypophysis of the dwarf mouse is only in a deep resting state (asleep) and is still able to respond to stimulation.

In comparing this hypophysis-to-hypophysis interaction, could a thymus-to-thymus interaction be acquired? The thymus also secretes several hormones. The answer is short and simple: We just do not know.

Thus, an intricate network of hormonal interaction appears to influence the immune functions. The notion of a coherent coordinative mechanism for these functions can be imagined. This suspicion could be supported further from the observations made on the nervous system's involvement in these interactions. Destruction of hypothalamic areas was followed by a depression of the immune response; their stimulation by faradization enhanced it [294]. Serum corticosterone level, action potential of the hypothalamic ventromedian area, and the number of plaque-forming cells in the spleen increased in demonstratively parallel curves following sheep erythrocyte injections in rats [54]. The stimulatory influence of antigens on these mechanisms seems to be exerted at different levels and not on the hypothalamus alone. Corticosterone production by incubated adrenals was increased, following addition of an antigen (enterotoxin) to the medium [224], and the corticotropin release of incubated hypophyses was also increased by the addition of *E. coli* endotoxin [491, 358].

The thymus may exert an influence on this mechanism. RNA synthesis increased in the hypothalamus of thymectomized rats incubated in vitro. This was prevented by thymosin or homeostatic thymic hormone injections. Added to the medium, both hormones caused a decrease in RNA synthesis in the incubated hypothalamus of intact rats [142]. This seems to indicate a negative feedback effect of thymectomy on the hypothalamus. This entirely new field awaits further research.

8 Possible Alimentary Influences

The picture of coordinatory mechanisms of immune functions is not complete if alimentary factors are not taken into account. The experimental documentation on this is poor. However, a superabundant number of clinical documentations are available concerning immune disturbances in underfed human subjects. Avitaminosis A, pantothenic acid, and pyridoxal deficiency in rats and avitaminosis C in guinea pigs resulted in a decreased antibody production. Thiamine, riboflavine, and folic acid deficiency were of no influence [see review 23]. Pair-fed controls showed a normal immune response, however, their thymus was also atrophic. Thus, this influence of specific deficiencies on the immune response did not result from the underfeeding which is by definition a consequence of avitaminoses. Rat fed a pyridoxine-deficient diet had no immunocompetent mature T cells in the thymus. Thymocytes from these rats acquired immunocompetence when incubated on a monolayer of thymic reticuloepithelium from normal rats, but not if the reticuloepithelial cells were taken from pyridoxine-deficient rats [540]. This suggests that pyridoxine deficiency may interfere with the immune response inasmuch as it inhibits thymic hormone production. This is perhaps not the only way. Lymphocytes from BALB mice incubated with irradiated C_3H lymphocytes developed cytotoxic functions, which decreased in time after the antigenic lmyphocytes were added after more than 20 days of culture. After 35 days of culture, the ability to develop cytotoxic functions was lost. It was restored by addition of pyridoxal to the medium [464].

Here again a new field of interest opens. As long as our documentation on this subject remains as poor as it is, the coordinatory mechanisms of immune functions will not be completely understood.

9 General Conclusions

This review contains evidence for the influence of nearly all hitherto known hormones on the immune response. As stated above, the documentation for this evidence differs quantitatively from one hormone to another: abundant for the thymus, in general it is scarce for the other endocrines, because the attention of immunologists has thus far focused more or less exclusively on the thymus. This is unjustifiable. There is valid evidence for pluriglandular influences on the immune response. *Komeva's* observations indicate an influence of the hypothalamus [294].

It was postulated for the thymus, that its influence is needed for the differentiation of the immune system (thymectomy is of no consequences in animals whose immune system is already mature). This is not entirely valid. Endocrine mutilation resulted in immune disturbances in prepuberal animals, as was shown above.

A difference must be made between mature lymphocytes and sentitized lymphocytes. In the lymphocytes, the first contact with an antigen results in changes which are under hormonal influences. Once these changes have occurred, hormones are apparently no longer needed (the secondary immune response is not influenced by endocrine mutilation.

The mechanism for these hormonal influences differs from one endocrine to another. Some act directly on the immune system. The synergic growth hormone, the thymus, and the corticosteroids are examples of this condition. Some endocrines influence the immune system inasmuch as they stimulate or inhibit other glands. This may occur in two different ways:

1. An interaction between two hormones in their circulating form (such as the interaction thymus-corticotropin)
2. An influence of a hormone on a gland itself; the influence of the thyroid and the gonads is mediated by the thymus.

A scheme (Fig. 1) summarizes those interactions.

Do these observations themselves justify the postulate of a hormonal coordination of the immune response? At first glance, no. It requires the demonstration of an induction of the hormonal reaction by a specific stimulant. The stimulant may act directly (the isolated perfused pancreas secretes increased amounts of insulin if the glucose content of the perfusion liquid is increased). The reaction may result from an intricate mechanism involving several organs (energetic metabolism increases in the cold, under the influence of the thyroid and the adrenal cortex, but not in hypophysectomized animals). In order to accept the postulate of a hormonal coordination of the immune response, we must observe, that the presence of an antigen within the organism results in an increased activity

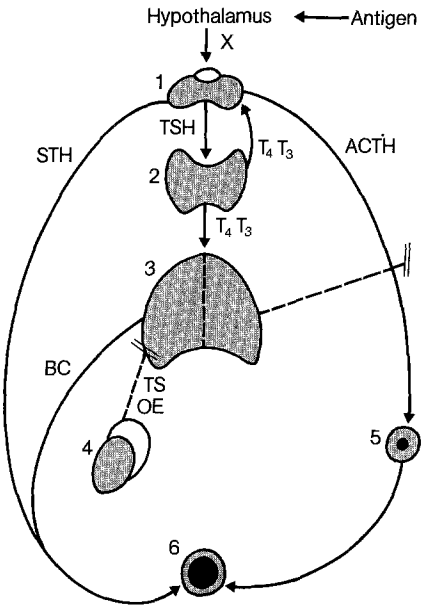

Fig. 1. Summary of the hormonal coordination of the immune response. → Stimulating influences, - - - - -// inhibiting influences. *1*, adenohypophysis; *2*, thyroid; *3*, thymus; *4*, gonads; *5*, adrenal; *6*, lymphocytes. *Abbreviations: BC*, thymic hormone *(Bernardi-Comsa); TS*, testosterone; *OE*, estradiol; *X*, unknown; *STH*, growth hormone; *TSH*, thyreotropin; *ACTH*, corticotropin; T_4T_3, thyroid hormone

of the endocrines mentioned above. This evidence is given partially by the observations of *Besedowsky* and *Sorkin* [54]: in presence of sheep erythrocytes there is a parallel increase of the action potentials in the hypothalamus, the corticosterone content of the blood, and the number of plaque-forming cells in the spleen. It would be of supreme interest to extend this fundamental observation to the other endocrines known to influence the immune system.

Be that as it may, our knowledge of these mechanisms will remain incomplete as long as alimentary factors are not taken into account. The observations of *Willis-Carr* and *St. Pierre* are of great interest in this context [540]. Earlier documents are not entirely satisfactory, concluding (in our opinion somwhat quickly) that most alimentary factors are of little or no influence on the immune system [see 23]. This conclusion is in striking contradiction to the everyday clinical routine. It is universally known that patients suffering from malnutrition are highly sensitive to infection that can develop with a peculiarly poor reaction and a catastrophal prognosis. This suggests the hypothesis of a state of immunodeficiency in these patients. To our knowledge, no attempt has yet been made to verify this hypothesis.

On the other hand, it is known that malnutrition results in an intricate hormonal deficiency. A characteristic pattern of thymic atrophy is an early consequence of every deficiency disease. Later, the adenohypophysis is severely affected (malnutrition was called the nonsurgical hypo-

physectomy). This, in turn, affects the thyroid, the adrenal, and the gonads. The documentation reviewed allows us to postulate a link between hormonal deficiency and immune deficiency (to some extent underfed subjects suffer from immune deficiency inasmuch as they suffer from hormonal deficiencies).

One may conceive that the thymus is of primary importance in this syndrome. Its atrophy is peculiarly early and pecularly severe. In dyspeptic infants underfed for therapeutic reasons the thymus may lose 90% of its normal weight and its hormone content tends toward zero [see 329]. In the late 1940s an attempt at substitutive therapy with thymic extract was made in these patients with encouraging results [see 329].

In our opinion, interesting new aspects of the physiopathology of the immune response may appear if:

1. The pluriglandular coordination of the immune response were to be considered (instead of the exclusive concentration on the thymus).
2. The accidental, acquired forms of immunodeficiency were also investigated (research has so far focused on congenital forms).

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