ADVANCES IN CLINICAL CHEMISTRY

Volume 15

Oscar Bodansky &
A. L. Latner

ADVANCES IN CLINICAL CHEMISTRY

VOLUME 15

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Advances in CLINICAL CHEMISTRY

Edited by

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Corbet Page Stewart

OBITUARY

Corbet Page Stewart

1897–1972

The death of Corbet Page Stewart on April 5, 1972 preceded by just a few days his 75th birthday. He was, along with the late Harry H. Sobotka, coeditor of *Advances in Clinical Chemistry* from Volume 1 in 1958 through to Volume 9, and continued in this capacity, along with Oscar Bodansky, until Volume 13 in 1970. There is no doubt that he played a very large part not only in the actual birth of this Serial Publication, but also in the standard which it has achieved. He brought with him an exceptionally wide knowledge of clinical chemistry, as well as an extraordinary facility for decisions regarding those people who were authorities in the advancing aspects of the subject. His facility as an editor was widely recognized. It was matched only by his ability to get on very well with colleagues and to render harmonious many situations which might otherwise have proved disruptive.

Corbet Page Stewart, affectionately known as "C.P." to his friends and colleagues, was born on the 14th April, 1897, at Willington, County Durham, where his father was the schoolmaster. He was educated at Bishop Auckland and subsequently at Armstrong College, Newcastle upon Tyne, which was, at that time, part of the University of Durham. He graduated in chemistry in 1920; his studies had, however, been interrupted by military service in the First World War. He subsequently studied for his doctorate under Professor George Barger in the Department of Medical Chemistry, University of Edinburgh, and proceeded to the Ph.D., his first doctorate, in 1925. He had held a Beit Memorial Fellowship from 1923 to 1925 and during this period worked each summer with Professor Gowland Hopkins at Cambridge. It was inevitable that, because of his publications and his international reputation, he proceeded to a second doctorate, the degree of D.Sc.

In 1926, he took up the appointments of Biochemist to the Royal Infirmary, Edinburgh, and Lecturer in the Department of Biochemistry of the University of Edinburgh. Such a joint appointment was, at the time, an unusual phenomenon, for his predecessor at the Royal Infirmary, Charles Harington, did not, in fact, hold a University appointment. Dr. Stewart gradually became a full-time clinical chemist and taught medical as well as science students.

From 1926 until the Second World War, he developed the biochemical service at the Royal Infirmary. At the same time he evolved undergraduate courses in medical biochemistry. In 1940, he was appointed Honorary Director of the Edinburgh and South-East Scotland Blood Transfusion Service and later became its Chairman. During the Second World War he was a member of the Medical Research Council's Blood Transfusion Research Committee and Adviser to the Polish Red Cross Institute of Blood Transfusion. As a result of this latter work, and his role in relation to the Polish Medical School, he received the honor of Polonia Restituta from the Polish Government-in-exile.

From 1942 to 1945, Dr. Stewart was a member of the Committee of the Biochemical Society.

In 1946, the University of Edinburgh established a Department of Clinical Chemistry with Dr. Stewart as Head and its first Reader. In 1948, with the onset of the National Health Service, he was made honorary consultant in clinical chemistry to the South-East Scotland Regional Hospital Board—an unusual event for a scientist who did not hold a medical degree. It was due recognition, however, of the remarkable knowledge of medicine in an individual without formal training in the subject. He was also appointed to the Board of Management of the Edinburgh Central Hospitals, and between 1956 and 1964 was Chairman of that Board as well as of the Boards of the Sick Childrens' Hospital and the geriatric hospital, Queensberry House.

In 1960, his Department at the Royal Infirmary, Edinburgh, moved into a new building, which was at that time acknowledged to be, and still is, one of the finest laboratories in the world. I visited it on a number of occasions and was more impressed each time. The move coincided with the 4th International Congress of Clinical Chemistry held in Edinburgh, with Dr. Stewart as Chairman of the Organising Committee. Three years later, in 1963, he served in a similar capacity at the International Congress on Nutrition, also held in Edinburgh. He was a member of the Organizing Committee of the Annual Colloquia on Protides of the Biological Fluids held at Bruges, and played an important role in relation to the West European Symposia on Clinical Chemistry.

Dr. Stewart was a leader in the development of clinical biochemistry in the United Kingdom. He was a founder member of the Association of Clinical Biochemists and became a member of its Council, subsequently its Chairman and eventually its President. I was, at that time, Chairman and together we drew up the first real Constitution of the Association. I well remember the wisdom he displayed both in this respect and later on in regard to the advice he gave me when I succeeded him as President.

His published work covered many fields and included diverse subjects such as the chemistry of amino acids and peptides, especially glutathione; mineral metabolism, with special reference to calcium; melanin pigment metabolism; ascorbic acid metabolism; metabolic aspects of cardiac muscle; and analytical techniques for lipids, nitrogenous compounds, and cortisol. He was an extraordinarily meticulous analyst who, from the first, maintained that the standards of technique in the service laboratory should be the same as those required for research purposes. He maintained that the fulfillment of clinical chemistry demanded equal collaboration between physician and chemist. The function of the latter was not to usurp that of the former but to assist the clinician by helping to shed light on the nature of an illness.

In addition to his large output of scientific papers, Dr. Stewart, was coauthor with D. Dunlop of *Clinical Chemistry in Practical Medicine* (E. & S. Livingstone Ltd., New York, 1st ed., 1931; 6th ed., 1962); with A. Stolman he was coeditor of *Toxicology Mechanisms and Analytical Methods* (Academic Press, New York, Vol. 1, 1960; Vol. 2, 1961). He was a member of the Editorial Board of *Clinica Chimica Acta* from the time of the foundation of that journal. He became Editor-in-Chief in 1960 and held this appointment until just before his death.

Stewart had many interests and talents outside the laboratory. As a youth he represented the University of Durham at cricket and hockey, and was a keen badminton player and an enthusiastic hill walker. In addition to being an excellent photographer with a keen eye for good composition, he had a fine collection of United States stamps, and was so interested in church architecture and history that he would make lengthy detours to add to the list of cathedrals and abbeys he had visited and about which he had an enormous store of knowledge.

There are very few men who will be remembered by their friends and colleagues with such deep respect and affection. Come wind, rain, or snow in any part of the world "C.P." would appear at meetings without hat or overcoat but with the inevitable cheroot or cigarette and a welcoming smile on his face. He obviously enjoyed life to the full and led a full life long into his retirement. In spite of his great ability, he was a very modest man, who achieved the highest pinnacle of success without blowing his own trumpet. He was always helpful to others, no matter how junior.

In 1963, Dr. Stewart received the Ames Award of the American Association of Clinical Chemists and in 1972, just before his death, he learned that he was to be the second recipient of the Distinguished Clinical Chemist Award of the International Federation of Clinical Chemistry. The award, presented after he had died, was received by his son, in the

presence of Queen Margrethe II of Denmark, at the opening ceremony of the 8th International Congress on Clinical Chemistry at Copenhagen on June 18, 1972. Those of us present will never forget this very moving ceremony and the intense applause when the award was received.

A. L. LATNER

PREFACE

In this volume of the Advances, the Editors have continued to follow the original dual aim of the series: the description of reliable diagnostic and prognostic procedures and the elucidation of fundamental biochemical abnormalities that underlie disease. As is true for so many other branches of science, clinical chemistry is experiencing an ever-accelerating pace of technological advance and accrual of new information. It is incumbent upon the clinical chemist to be aware of these changes, and to choose the particular technology and acquire that information which best suits the needs of his particular situation.

In their review on instrumentation in clinical chemistry, Broughton and Dawson have treated most comprehensively the principles underlying the use of various types of instruments in clinical chemistry, envisioning the incorporation of such instruments into automated and computerized systems. Scott has discussed a relatively new type of technology, namely, automated, high resolution analyses by liquid column chromatography. He describes procedures by means of which a large number of the constituents of a sample mixture are separated and quantified. Huisman reviewed the subject of normal and abnormal hemoglobins in these Advances in 1963, but the past nine years has seen such progress in various aspects of this important field that it was deemed advisable to bring the subject up to date. Although the enzyme acid phosphatase was discovered in 1925 and claimed considerable attention in the thirties and forties, no review of the entire subject has heretofore appeared in these Advances. Bodansky has considered not only the generally appreciated role of this enzyme in diagnosis of cancer of the prostate, but has also reviewed more recent applications in other diseases, in genetics, and in general biology. The metabolic responses following surgery or other physical trauma have been of substantial interest for several years and Johnston has now reviewed in some detail the endocrine aspects of these responses.

As in the past, it is a great pleasure to thank our contributors and publisher for their excellent cooperation in making this volume possible.

OSCAR BODANSKY
A. L. LATNER

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AUTOMATED, HIGH-RESOLUTION ANALYSES FOR THE CLINICAL LABORATORY BY LIQUID COLUMN CHROMATOGRAPHY

Charles D. Scott

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1. Introduction

Many analytical methods used in the clinical laboratory today result in the analysis of a single constituent or of a single group of constituents in a physiological sample mixture. In most of these analytical procedures, an attempt is made to quantify the constituent without isolating it from the complex mixture. A great deal of developmental effort has been directed toward mechanizing many of these methods and, in some cases, in combining several analyses into a single, complex, automated instrumental array that requires a minimum of operator time. Although this

[1] Operated for the U. S. Atomic Energy Commission by Union Carbide Corporation.

developmental work has been extremely important to the clinical laboratory from the standpoint of economics, recent research in the medical sciences will probably lead to even more drastic changes in the clinical laboratory in the near future.

It is now apparent that many pathological states will ultimately be defined, studied, and treated on the molecular level. There is a considerable body of information that suggests that the levels of chemical constituents in various body fluids can be used to help indicate bodily function and malfunction. This is not a new concept for the clinical laboratory, but the number of these potential "chemical indicators" has been expanded to several hundred. For example, in a recent bibliography (K1) on urinary constituents, the literature for a three-year period has over 3000 citations to over 700 molecular constituents, many of which could have pathological significance.

Quantitative methods for analyzing for large numbers of the individual constituents of body fluids have frequently involved several steps and excessive operator time. As a result, such complex analyses have been relegated to the research laboratory. It would be extremely difficult and expensive for the clinical laboratory to use these methods on a routine basis, even if they could be entirely automated. However, new high-resolution analytical systems that are capable of automatically analyzing for many of the individual constituents of a physiological sample may be useful in the clinical laboratory for such an in-depth analysis.

The term "high-resolution analysis" has been chosen to describe an analysis in which a large number of all the constituents of a sample mixture are separated and quantified. Thus, high-resolution analytical techniques have two very necessary components: (1) a means of separating the individual components; and (2) a means of detecting and quantifying the separated components. In general, the separation techniques that have proved most satisfactory have been some form of chromatography or electrophoresis, and quantification has been achieved primarily by photometric monitoring for liquid systems and flame ionization for gaseous systems.

Relatively few truly automated, high-resolution analytical systems are now used in the clinical laboratory. For this presentation, I have arbitrarily chosen only those systems that use column chromatography for separation. This choice is based not only on the ability of these systems to separate literally hundreds of the molecular constituents in a physiological fluid but also because they are directly amenable to a high degree of automation. Obviously, this latter point is extremely important for any future development in the clinical laboratory. Further, only liquid chromatography will be discussed here since there has recently

been an excellent review of the use of gas chromatography in the clinical laboratory (S10).

It is difficult to establish the time, places, and pertinent investigators involved in developing high-resolution analytical systems based on liquid chromatography since this technology has been evolving for many years. Yesterday's high-resolution systems are now considered very low-resolution systems indeed. Certainly the early work of Cohn in separation of nucleic acid derivatives by ion-exchange chromatography (C2) was important, as was the development of an automated analytical system for amino acids by Moore and Stein (M1). Hamilton showed that literally hundreds of ninhydrin-positive compounds in urine could be separated and quantified by a modified amino acid analyzer (H3), and Anderson and others followed through on some of Cohn's work to automate the analyses of complex biological fluids in a single system (A1, S1).

There are at present many investigators involved in the general area of high-resolution analysis for the clinical laboratory. Many recent contributions in this field can be found in the proceedings of the annual symposium series on "High-Resolution Analyses and Advanced Analytical Concepts for the Clinical Laboratory" (S4, S6, S8).

2. Analytical Systems

Although the concentrations of the constituents of all types of body fluids represent potentially useful diagnostic information, analysis of the most complex body fluid, urine, presents the most ambitious challenge. One of the most severe tests for the utility of a high-resolution system is its usefulness in analyzing for the constituents of urine. This body fluid has long been neglected in the clinical laboratory. The four analytical systems that will be considered here are at least potentially useful for urine analysis as well as for the other less complex body fluids. They are primarily used for the analysis of the low-molecular-weight (less than 1000) constituents.

Two of these systems, an analyzer for the UV-absorbing constituents (UV-analyzer) and one for carbohydrates, will be discussed in some detail. Two others, one for ninhydrin-positive compounds (amino acids and related compounds) and an analyzer for organic acids, will be introduced as systems that have great potential but which have not been fully developed as yet. These four analytical systems certainly do not represent all the concepts for the use of liquid chromatography in body fluids analysis; however, they are systems that have been used at least to some degree in clinical and medical research laboratories.

The UV- and carbohydrate analyzers were specifically developed to

be used for analyzing body fluids, and prototype systems of each analyzer are now being used at several laboratories. On the other hand, the ninhydrin-positive and organic acid analyzers were not originally developed to be used for complex body fluids, but rather for much simpler mixtures, e.g., protein hydrolyzates. As a result, these two systems have not been fully exploited for body fluids analyses, particularly for urine analysis, although preliminary work indicates that they may have great utility. Thus, the latter two systems will not be discussed in as much detail as the UV- and carbohydrate analyzers.

3. Description of Analyzers

Up to this point in time, high-resolution liquid chromatography requires the use of very small sorption particles packed in relatively long columns. This results in the necessity of operating with relatively high column inlet pressures to force the eluent through the column at a reasonable rate. This requirement of high-pressure operation is the major difference between high-resolution systems and the more conventional liquid chromatography. Much of the following discussion will emphasize the high pressure requirements.

3.1. General System Description

Automated liquid chromatographs contain the following major components: (a) the separation section, which consists of a closed tubular column packed with small particles of the solid sorbent or support material; (b) an eluent storage and, in some cases, an eluent gradient preparation section; (c) an eluent delivery system equipped to deliver the eluent to and force it through the separation column; (d) a means for introducing the sample to the column; and (e) a means for detecting and quantifying the separated constituents in the column eluate (see Fig. 1). Automated data acquisition and processing may also be used.

The requirements of high-pressure operation affect the design and operation of the eluent delivery, sample introduction, and separation systems. Many of those involved in developing high-resolution analytical systems for body fluids have made very significant contributions to high-pressure liquid chromatography technology.

3.2. Separation Systems

The most important component of the liquid chromatograph is the separation system. Recent advances in liquid chromatography have included the development of many new types of sorption media that have made high-resolution separations possible.

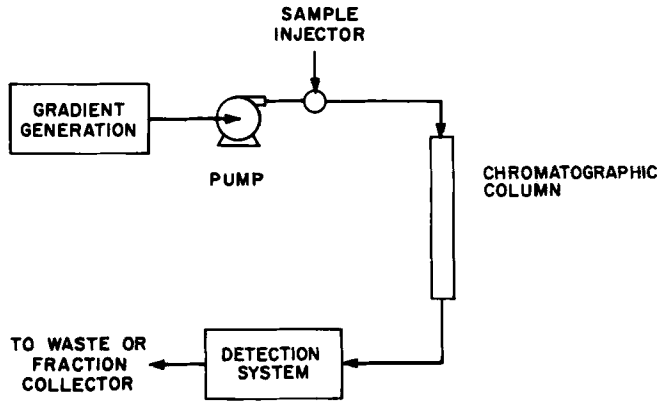


FIG. 1. Liquid column chromatography.

3.2.1. *Separation Media*

The aim in recent developments has been to produce media in which the solid-phase mass transport resistances are reduced. A reduction in these resistances will allow the chromatographic system to operate closer to equilibrium conditions, and should result in faster and more effective separations. All the systems under consideration here achieve high resolution by using relatively small particles (down to about 10 μ diameter) in the stationary sorption phase in chromatographic columns up to about 150 cm long. The small particles are used to reduce the solid-phase diffusional effects, and the relatively long columns are necessary to provide a sufficient number of separation stages to achieve the high resolution.

3.2.2. *Pressure Drop*

The combination of small particles and long columns contributes to high operating pressures. The effects of column and operating parameters on the pressure drop of liquid-chromatography columns designed to operate at pressures less than about 100 psi can essentially be disregarded since design problems are minimal; however, these effects become very important in high-pressure chromatography (greater than 1000 psi). For a particular type of sorption medium, the major parameters that influence the pressure drop across an ion exchange column are: particle diameter, flow rate, column length, and fluid properties such as density and viscosity. These effects have not been thoroughly studied for small particles; however, previous data (H2) and some of the author's recent work have shown that the pressure drop across a packed column is inversely dependent on the square of the mean diameter of

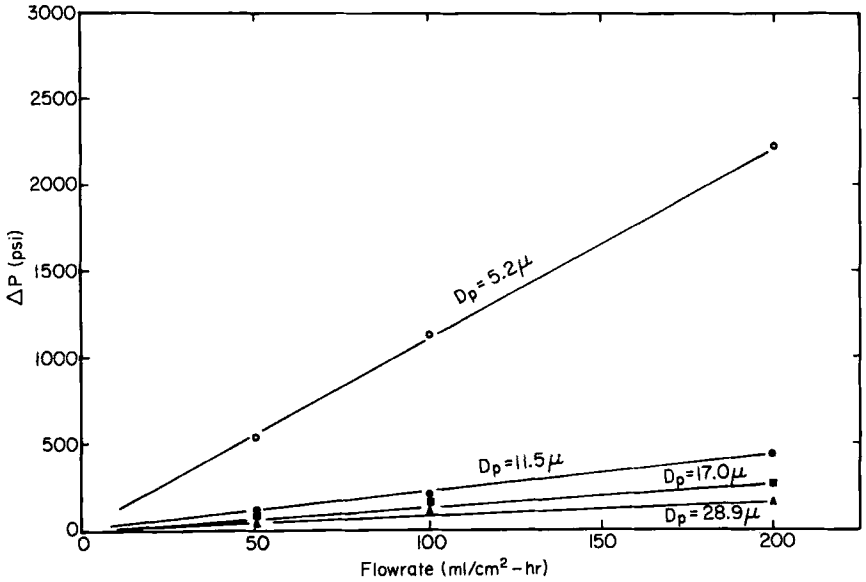


FIG. 2. Pressure drop across ion exchange resin columns as a function of flow rate for resins of different particle size. Operating conditions: 40°C; column, 0.62 × 100 cm, stainless steel; resin, Dowex 1 × −8.

ion exchange resin particles and linearly dependent on the linear velocity of the liquid phase and the length of the column (Fig. 2).

3.2.3. *Columns*

Metal columns, which can be easily fabricated from seamless metal tubing, can be used for high-pressure techniques. Conventional compression tubing fittings can be used for the fluid entrance and exit and for holding a porous metal support for the fixed bed (Fig. 3). Although the use of precision-bore tubing may be slightly more advantageous, good results have been obtained with common seamless tubing. Some glass columns operable to about 1000 psi are available and have been used in early models of the systems under consideration.

3.2.4. *Column Geometry*

The geometry of a chromatographic column has a significant effect on the resolution that is achieved. As the length of a column is increased, the separation of two components becomes more efficient; however, the width of the peaks is also increased. The diameter of the column should not have a great effect on resolution (assuming that comparable flow velocities and a proportionally scaled sample size are used) as long as

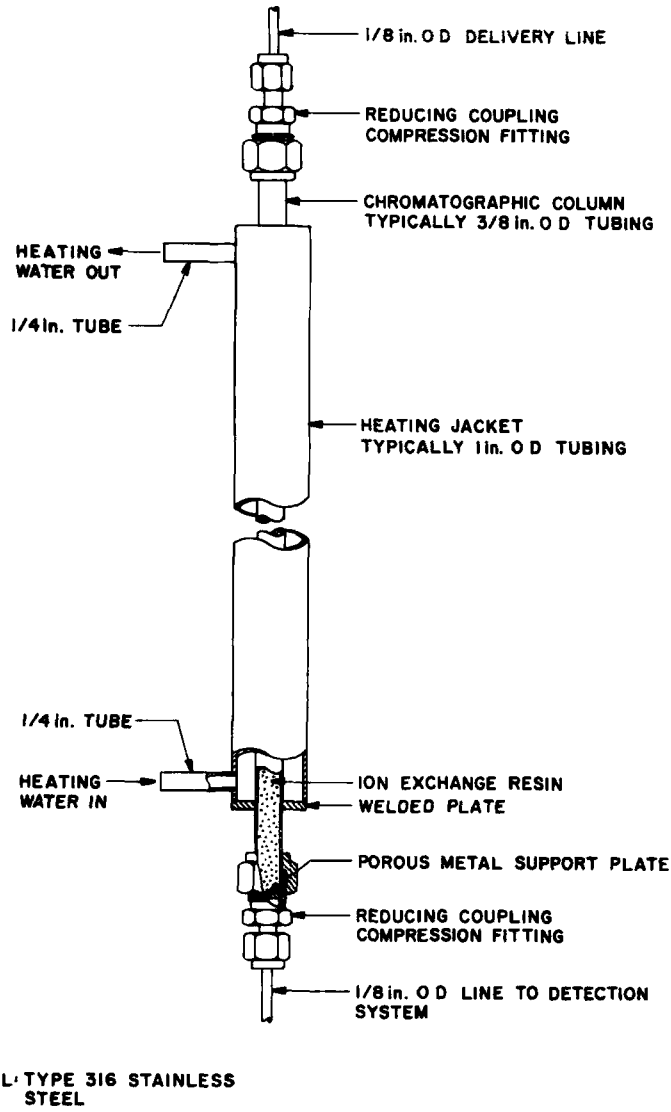


FIG. 3. High-pressure chromatographic column fabricated from stainless steel tubing. From Scott (S12) copyright © 1968 *Clinical Chemistry.*

the column is sufficiently small to prevent radial variations in fluid properties but not small enough to require a sample of such limited volume that the separated solutes cannot be detected by the column monitoring system. Column diameters in the range of 0.15 to 0.60 cm

have been found suitable for analytical purposes. Column lengths up to 200 cm have been used effectively.

3.3. Eluent Delivery

Two basic types of eluent delivery systems are used in liquid column chromatography. These are constant-flow devices and pulsating pumps (Fig. 4). Examples of the former include constant-drive syringes and reservoirs with gas overpressure, and the latter include reciprocating piston pumps. All the systems described here have been designed to use piston pumps with pulsating flow, although it would be possible to design such systems with constant-flow devices. It should be pointed out that in systems with a column pressure drop in excess of 1000 psi, pulsating pumps are sufficiently accurate metering devices with flow variations of less than 10% during each pulse cycle.

In general, pulsating pumps are less expensive and somewhat more simple to use in chromatographic systems. They are particularly advantageous when gradient elution (i.e., an eluent composition that changes

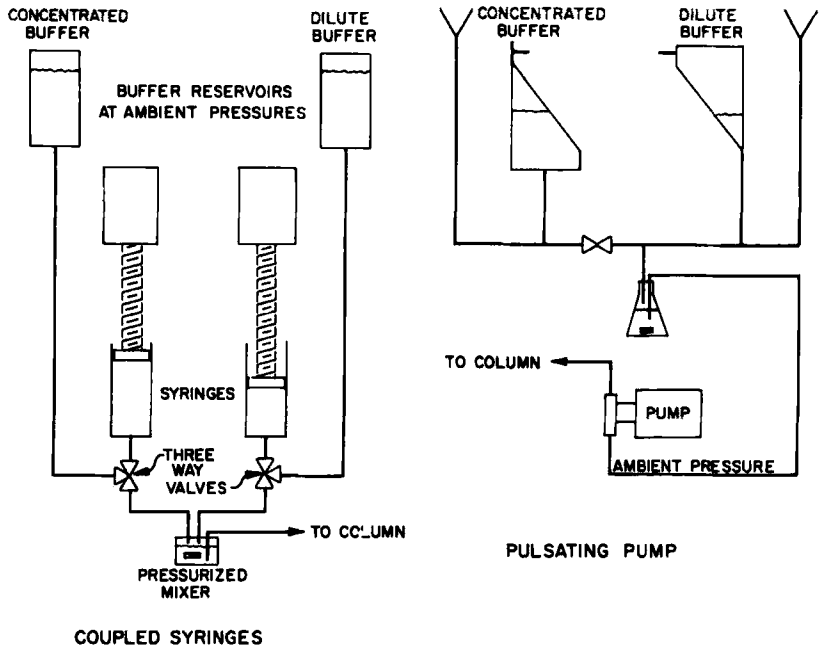


Fig. 4. High-pressure eluent delivery with gradient elution using coupled syringes or a pulsating pump.

with time or elution volume) is used, since the gradient can be developed prior to contact with the high-pressure environment (Fig. 4).

At pressures above about 3000 psi, it is difficult to maintain a good mechanical seal around a moving piston. The difficulties are usually more pronounced for the pulsating pump since its plunger moves more rapidly and more frequently than the constant-flow devices. This disadvantage has now been partially circumvented by the development of the diaphragm-plunger pulsating pumps in which a pulsating plunger delivers a hydraulic fluid to a sealed diaphragm in contact with the eluent. The eluent is pumped by the movement of the diaphragm, and this arrangement abolishes the need for a high-pressure seal. Such pumps are used successfully in the UV- and carbohydrate analyzers.

3.4. Generation of the Eluent Concentration Gradient

Gradient elution chromatography is a very powerful and frequently necessary technique when complex mixtures are being separated. Increasing the concentration of a buffer with time or elution volume decreases the distribution coefficients of the more strongly sorbed species, thus allowing the elution time to be significantly decreased without jeopardizing the separation of the less strongly sorbed species at the beginning. Changing the pH or some other eluent property also allows a more efficient separation.

Nearly any type of continuous eluent gradient can be generated by connecting two or more chambers containing solutions of different properties to a common mixing chamber (Fig. 4). (See also the description of UV- and carbohydrate analyzers.)

The eluent properties of the fluid stream from such a system vary with the volume removed, depending only on the relative cross-sectional areas of the chambers and the properties of the fluid being used as the eluent. Typically, operation is initiated by filling each chamber until overflow occurs. Then, as the run progresses, the eluent properties change due to the changing cross-sectional areas of the chambers. At the end of the run, a reservoir connected to the bottom of the chamber containing the initial eluent automatically equilibrates the column with the starting eluent in preparation for the next run.

A stepwise eluent gradient can be generated by simply using a series of reservoirs with different eluent solutions all connected to the pump feed line and each line being actuated by a solenoid valve. (See description of the ninhydrin-positive compound analyzer.) This technique works well if the step changes do not upset the monitoring device; however, it necessitates additional equipment.

3.5. Sample Introduction

The most effective method for introducing a sample into an automated chromatographic system is to feed it directly into the eluent line just before the latter contacts the chromatographic column. A hypodermic syringe entering a septum connected to the eluent line may be used to accomplish this; however, in high pressure operation this will usually necessitate stopping the eluent flow so that the septum and syringe are exposed to a reduced pressure.

The UV- and carbohydrate analyzers use a sample injection valve that contains six ports, each pair of which is interconnected. In one orientation of the valve, a sample can be loaded into the sample loop, which becomes a part of the eluent line when the ports are reoriented (by turning the valve handle) (Fig. 5). Valves that allow automated sample introduction at pressures up to 5000 psi without interrupting the eluent flow have been developed and are now available commercially (S2).

3.6. Column Monitor

In all four systems, the eluate stream transports the separated constituents of the sample mixture to flow monitors that are either a photometer (for the UV-analyzer) or a colorimetric detector (for the other systems). In the latter case, reagents are mixed continuously with the

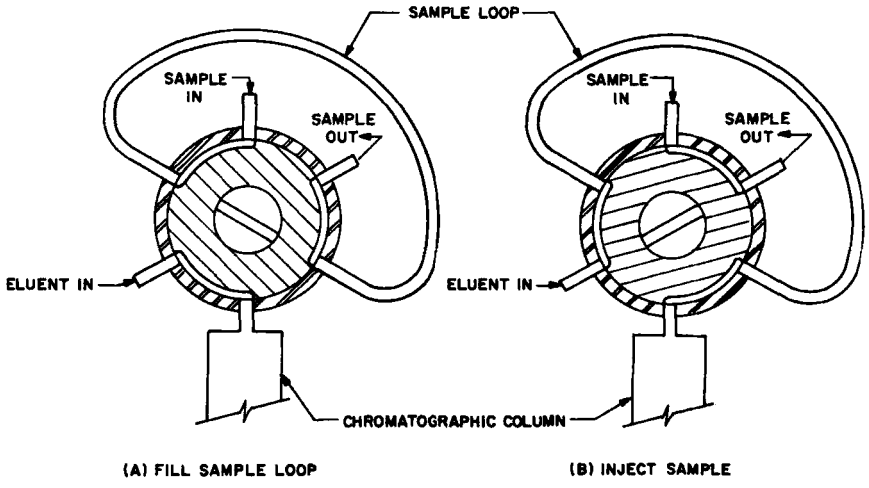


Fig. 5. Use of a six-port valve to inject a sample into the eluent stream of a chromatograph. From Scott (S11) with permission.

eluate stream and the resulting reaction mixture is continuously monitored by a flow colorimeter.

When colorimetric monitoring is used, additional process variables have to be considered. These result from the necessity of introducing a metered stream or streams of reagent into the eluate stream, mixing the two streams thoroughly, allowing the necessary chemical reaction to occur between the separated constituent and the reagent, and continuously monitoring this reaction stream with a colorimeter. For systems in which large reagent flow rates (greater than 10 ml/hr) are used, this can be done by metering the reagent streams with positive displacement pumps. When pulsating pumps are used, the variation in flow rates must be reduced by suitable damping devices.

For systems that require very low reagent flow rates, and even for larger flow rates, a successful reagent metering system can be designed to include a reagent reservoir with near-constant overpressure or hydrostatic head coupled with a controlled flow resistance, for example, narrow bore tubing or a control valve (J1). Rotameters can be used to monitor the actual flow rate. If the reagent hydrostatic head or gas overpressure remains essentially constant during the course of a run, the reagent flow rate will remain relatively constant even at a flow rate of a few milliliters per hour.

3.7. Data Reduction

All the systems discussed here use conventional strip chart recorders for recording the photometer or colorimeter output, and the resulting record is a conventional histogram in which the absorbance of the eluate or eluate-reagent reaction mixture is recorded as a function of time. In addition, some prototype systems of the UV- and carbohydrate analyzers use on-line computers for data storage and processing (C1, S7).

In any case, the area of each chromatographic peak is directly related to the quantity of material represented by that peak. Quantification of the chromatographic data is achieved either by graphical (strip chart recorder) or numerical (on-line computer) integration of each chromatographic peak to obtain the peak area. Where there are mutually interfering chromatographic peaks, the resulting absorbance envelope must be convoluted into its individual peaks. This is most easily done by the on-line computer using conventional spectral stripping techniques (C1, S7).

3.8. UV-Analyzer

The present model of the UV-analyzer will provide the basis for analytical systems that can be used routinely in the future (P1, S5).

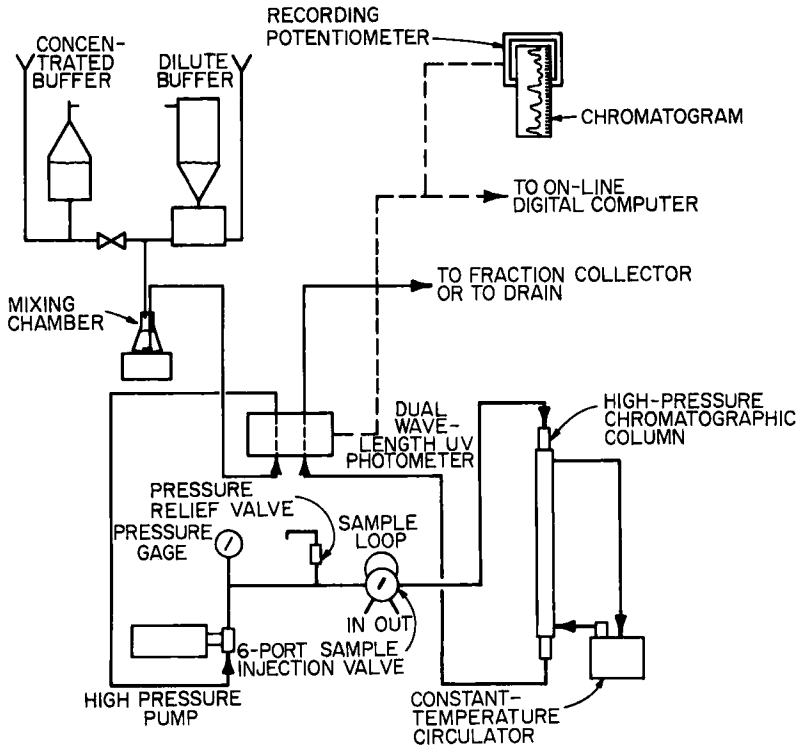


FIG. 6. Automated, high-resolution chromatograph for analyzing for the UV-absorbing constituents in body fluids.

Several prototypes of this analyzer are currently being tested at various clinical and medical research laboratories.[2]

The analyzer uses a heated, high-pressure (up to 4000 psi) anion exchange column, concentration gradient elution with an aqueous acetate buffer for separation and transport of the constituents of the sample mixture, and a recording photometer for detection and quantification of the separated constituents (Fig. 6). Earlier models of this analyzer were housed in standard 24 × 24 × 63 in. cabinets (Fig. 7); however, miniaturized versions with capillary separation columns are now being used (Fig. 8). An anion exchange resin produced by Bio-Rad Laboratories (Aminex A-27) in the size range of 10–15 μ has been found to be satisfactory.

[2] Construction prints of the earlier models are available as CAPE-1753 from the National Technical Information Service, U. S. Department of Commerce, 5285 Port Royal Road, Springfield, Virginia 22151.

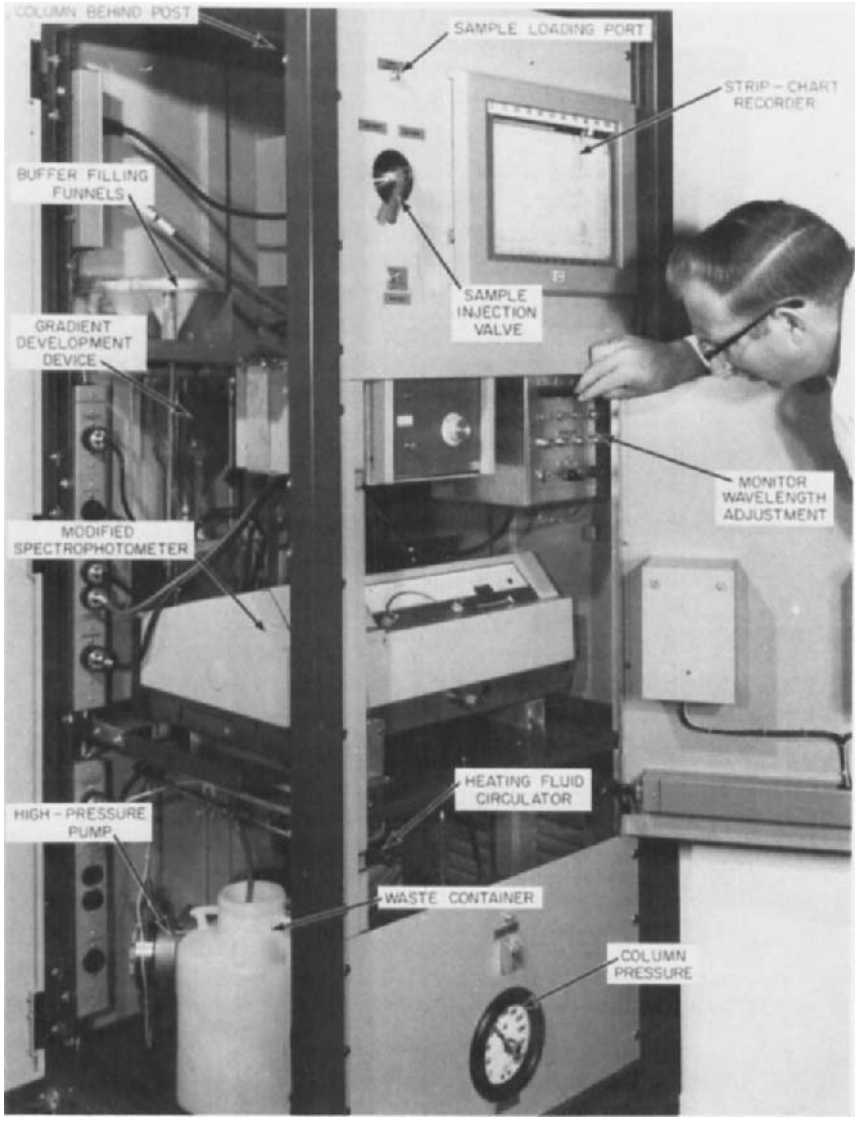


FIG. 7. UV-analyzer prototype Mark II. From Scott (S5) with permission.

The separation columns are fabricated from standard type 316 stainless steel tubing that is either 0.22 or 0.62 cm ID (depending upon whether it is an advanced miniaturized system or an earlier model) and 150 cm long. A 1 in. OD stainless steel heating jacket surrounds the column. The ion exchange resin is packed into the column as a thick slurry using a dynamic loading technique which provides reproducible

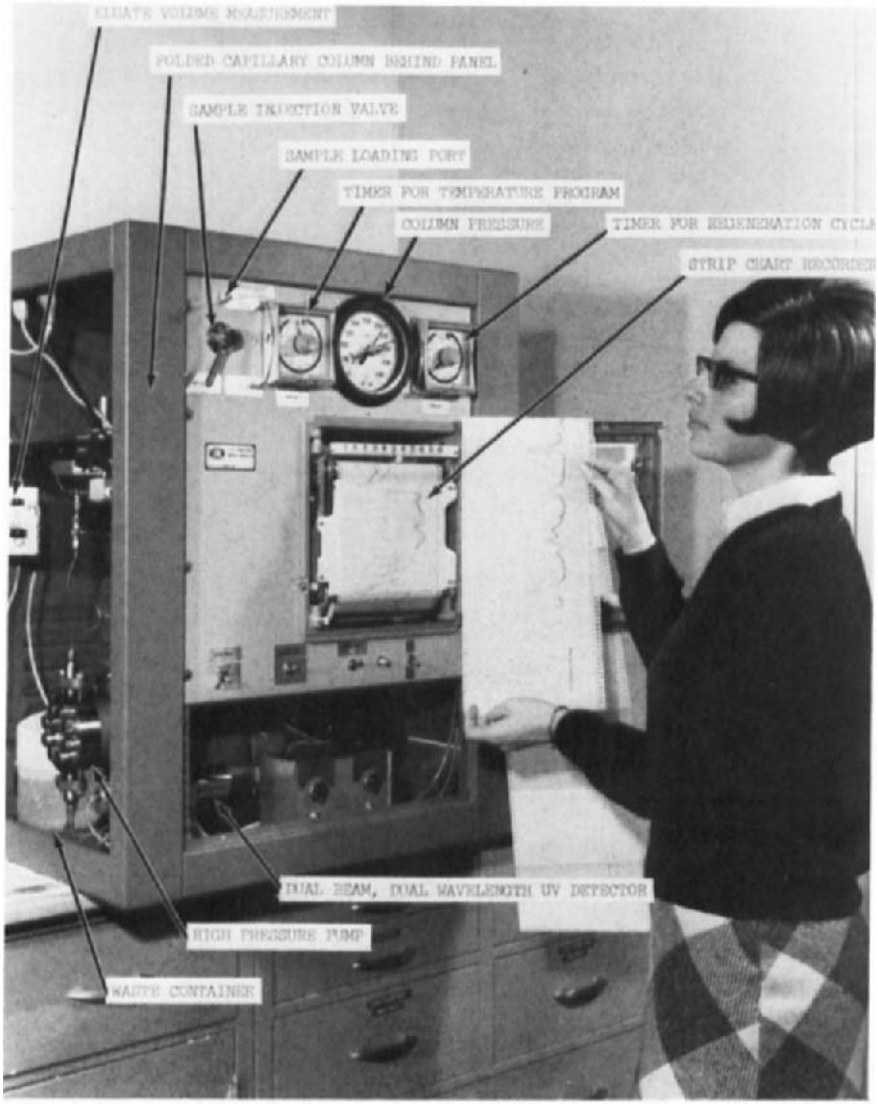


FIG. 8. Miniaturized Mark III-A UV-analyzer. From Pitt (P1), copyright © 1970 *Clinical Chemistry*.

loading from column to column (S3). An ammonium acetate–acetic acid buffer (pH 4.4) whose concentration varies from 0.015 to 6.0 *M* during the course of the analysis is used as the eluent, and the separation column is maintained at 25°C for the first 30% of the run and at 60°C thereafter by a heated circulating fluid.

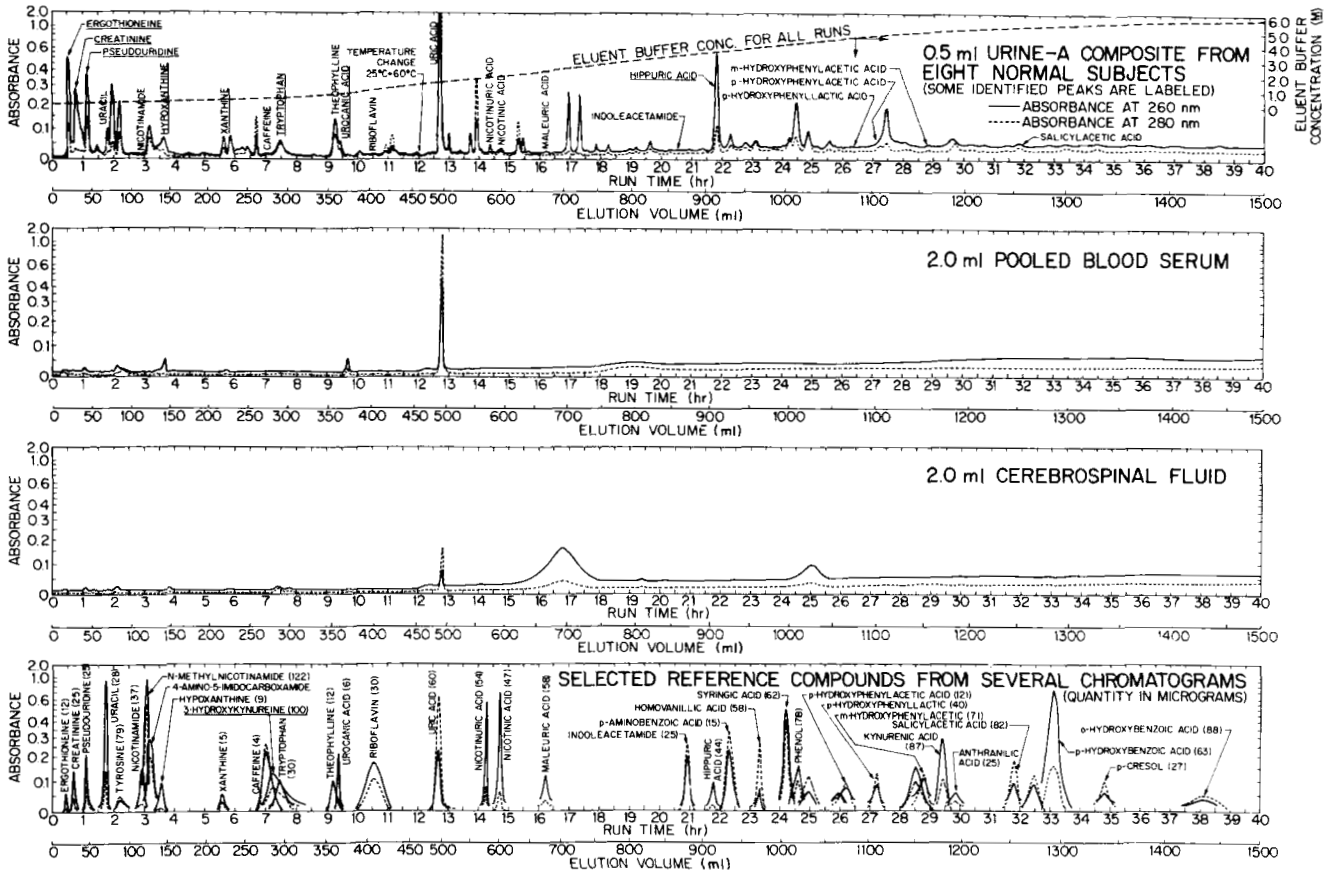


FIG. 9. Typical chromatograms from the Mark II UV-analyzer showing the difference between urine and other body fluids and the identification of some of the chromatographic peaks. From Scott (S5) with permission.

The detector is a miniature, recording, dual-beam UV flow photometer operating continuously at two different wavelengths, 254 and 280 nm (T1, T2). The dual-beam mode of operation provides a means of referencing the changing properties of the eluent stream by differentially comparing the eluent stream to the eluate stream.

Samples are introduced by a six-port injection valve, and analytical results are presented graphically as a chromatogram showing the UV absorbance of the eluate stream versus run time, each molecular constituent being represented by a chromatographic peak (Fig. 9). The required sample size is 0.1–0.5 ml, and the total separation time is 40 hours for the larger system and 24 hours for the miniaturized system. Sensitivity is a few nanograms for many constituents (Fig. 10).

3.9. Carbohydrate Analyzer

The carbohydrate analyzer also uses a heated, high-pressure anion exchange column of the same design and utilizing the same resin as that used for the UV-analyzer; concentration gradient elution with a borate aqueous buffer; and detection and quantification by a continuous colorimetric system (Figs. 11 and 12) (K2, S5).[3] Miniaturized versions using capillary columns are also now being used.

The borate buffer is necessary to complex the neutral carbohydrates to give them ionic properties that then allow separation by anion exchange chromatography. A sodium tetraborate-boric acid buffer (pH 8.5) whose composition varies from 0.169 to 0.845 M in the borate ion is used as the eluent. The anion exchange separation column is maintained at a constant 55°C.

Carbohydrate detection is by the continuous colorimetric reaction of sulfuric acid and phenol with the carbohydrates in the eluate. To accomplish this, the system includes: (1) a reaction column into which the eluate and reagents (5% phenol solution and concentrated sulfuric acid) are continuously metered and mixed; (2) a reaction section maintained at 100°C through which the reaction mixture flows; and (3) a flow colorimeter that continuously measures the absorbance of the reaction mixture at wavelengths of 480 and 490 nm (Fig. 11). The reagents are metered into the reaction column by using controlled pressure or hydrostatic head in the reagent reservoirs, a fixed pressure drop across a length of capillary tubing, and a control valve in the reagent lines (J1). Rotameters are used to measure the reagent flow rates.

[3] Construction prints of the earlier models are available as CAPE-1779 from the National Technical Information Service, U. S. Department of Commerce, 5285 Port Royal Road, Springfield, Virginia 22151.

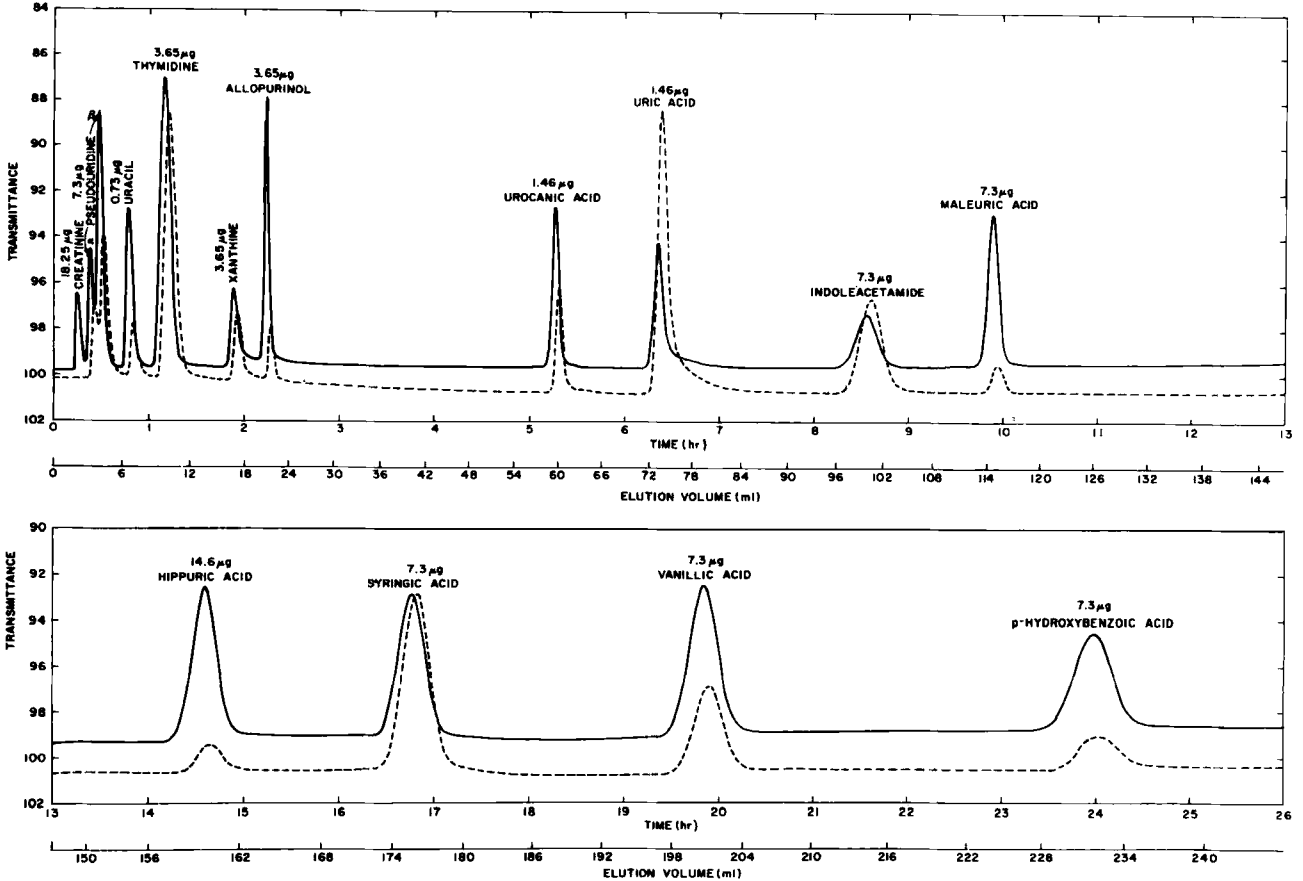


FIG. 10. Typical chromatogram from the Mark III-A bench-top UV-analyzer showing separation of small amounts of selected reference compounds. From Pitt (P1), copyright © 1970 *Clinical Chemistry*.

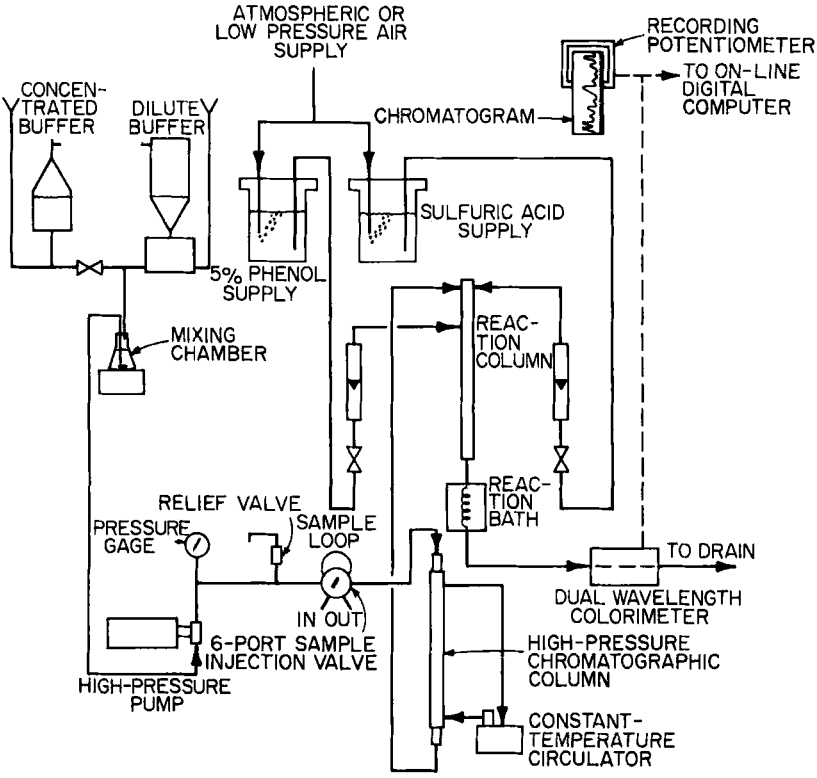


FIG. 11. Automated, high-resolution chromatograph for analyzing for carbohydrates in body fluids.

Samples of 0.5 to 12 ml are introduced by a six-port injection valve, and the resulting chromatogram is a measure of the absorbance of the eluate reaction mixture as a function of time (Fig. 13). Separation time is 20 hours.

3.10. NINHYDRIN-POSITIVE COMPOUND ANALYZER

The modern amino acid analyzer is one of the most highly developed liquid chromatographs now being routinely used in the research laboratories. It is also used to some extent for analysis of physiological fluids, mainly serum (E1). However, the resolution of such systems does not approach that which has been previously demonstrated, especially for urine analysis. Such a high-resolution analyzer has a great potential for the clinical laboratory.

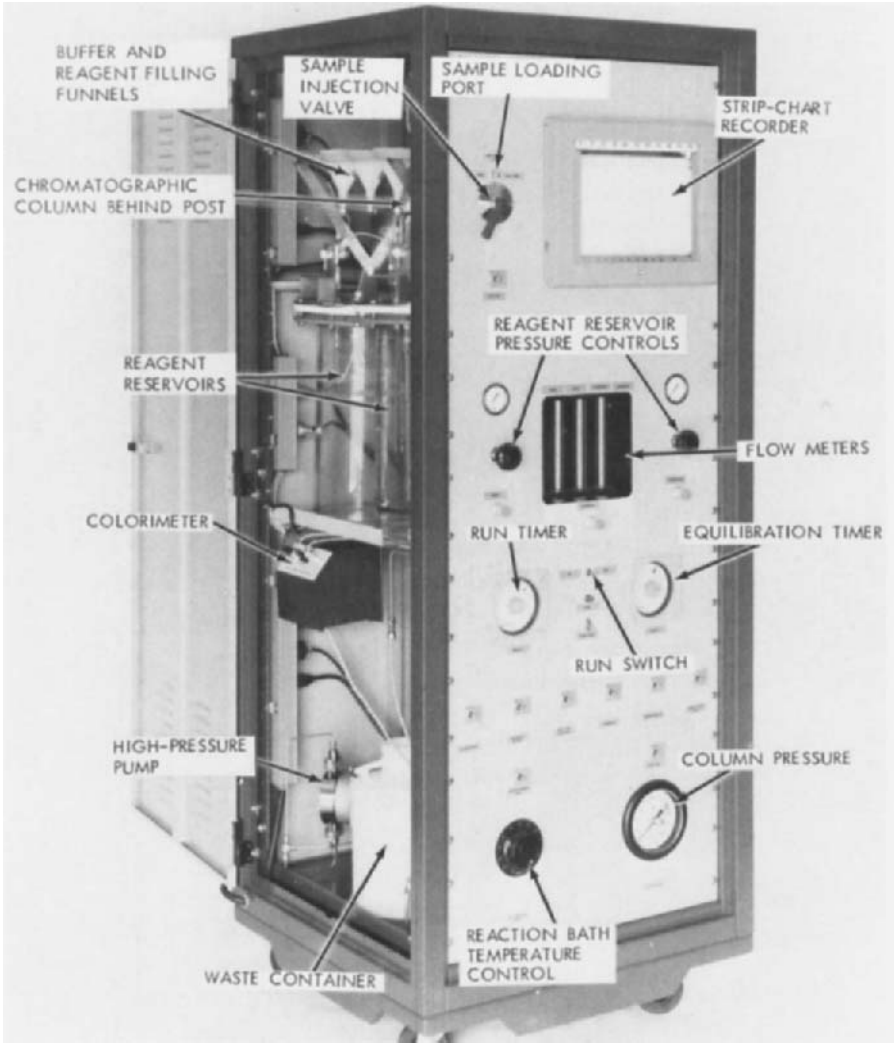
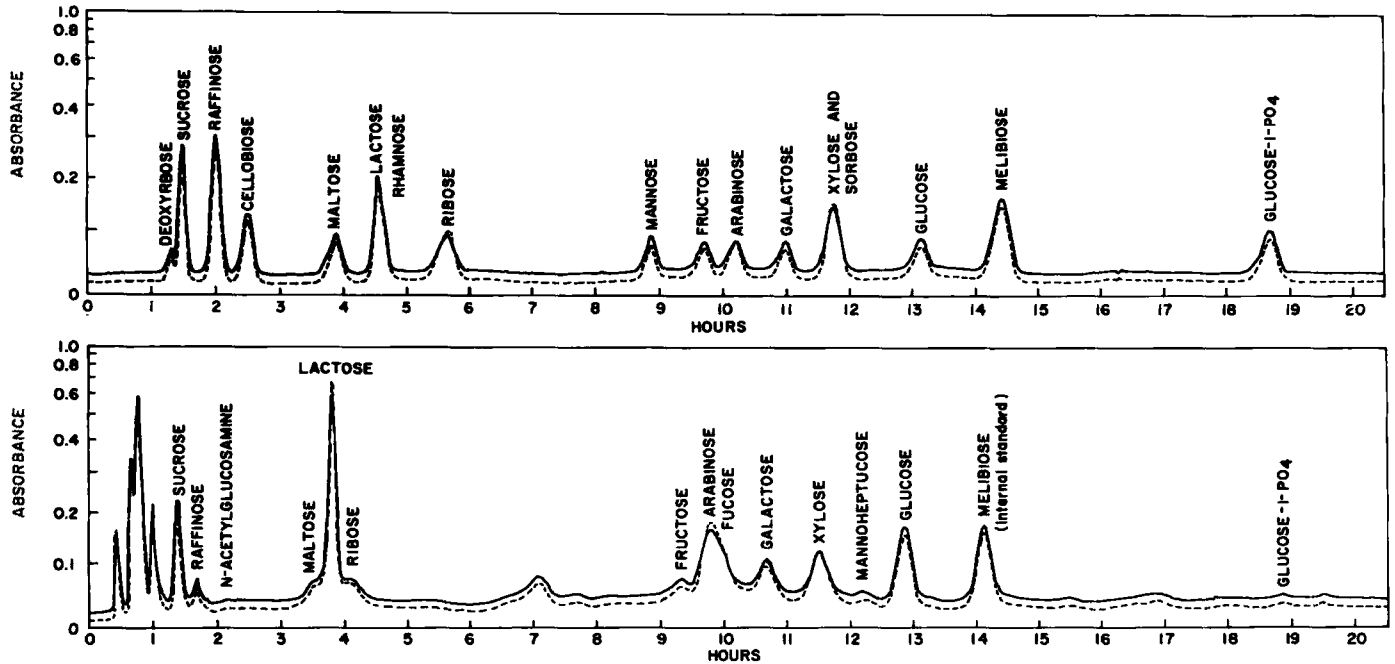


FIG. 12. The Mark II carbohydrate analyzer. From Scott (S5) with permission.

Many different experimental systems for analysis of amino acids have been described, but the most successful from the standpoint of high-resolution analysis of physiological fluids is the system described by Hamilton in which he was able to separate at least 175 components in human urine (H3) using a single cation exchange column system. This


ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0
DEOXYRIBOSE
SUCROSE
RAFFINOSE
CELLOBIOSE
MALTOSE
LACTOSE
RHAMNOSE
RIBOSE
MANNOSE
FRUCTOSE
ARABINOSE
GALACTOSE
XYLOSE AND SORBOSE
GLUCOSE
MELIBIOSE
GLUCOSE-1-PO4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
HOURS
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0.1
0
LACTOSE
SUCROSE
RAFFINOSE
N-ACETYLGLUCOSAMINE
MALTOSE
RIBOSE
FRUCTOSE
ARABINOSE
FUCOSE
GALACTOSE
XYLOSE
MANNOHEPTUCOSE
GLUCOSE
MELIBIOSE (internal standard)
GLUCOSE-1-PO4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
HOURS

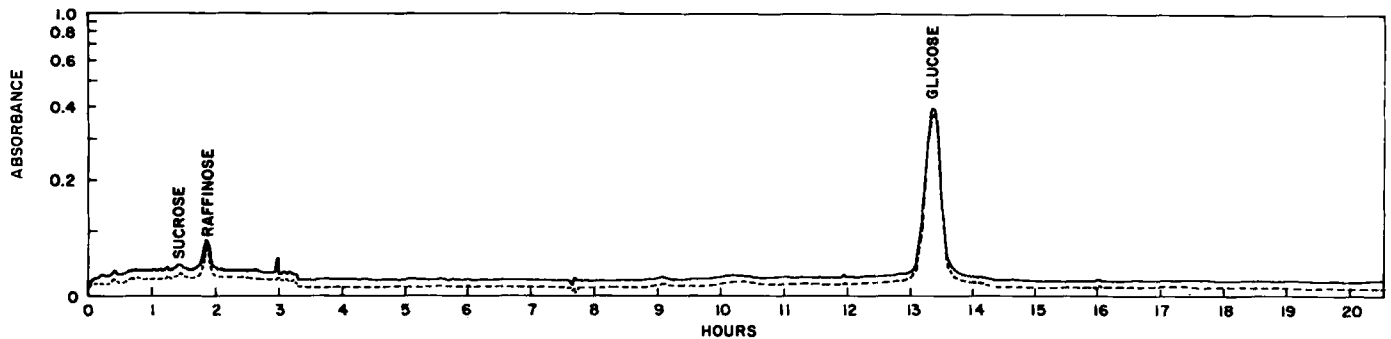


FIG. 13. Typical chromatograms from the carbohydrate analyzer showing the difference between urine and blood serum and the identification of some of the chromatographic peaks. Sample sizes: sugar reference compounds (*top*), 0.62 μM except 1.25 μM melibiose and glucose-1-PO_4; urine (*middle*), 12.4 ml; and blood serum (*bottom*), 1.6 ml (—— 490 nm, ———— 480 nm.) From Scott (S5) with permission.

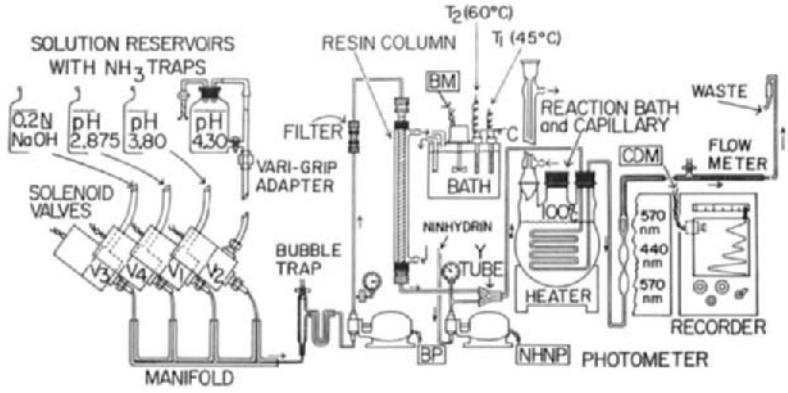


FIG. 14. High-resolution cation exchange chromatography of ninhydrin-positive compounds in body fluids. From Hamilton (H1), with permission.

system is composed of a high-pressure glass column 0.636 × 135 cm containing the ion-exchange resin that is temperature controlled by circulating fluid; a positive displacement piston pump for eluent delivery, with stepwise buffer change being controlled by a series of solenoid valves connected to the pump inlet manifold; and a ninhydrin colorimetric development system in which the ninhydrin-positive compounds in the column are reacted with a stream of a ninhydrin reagent followed by colorimetric monitoring at 440 and 570 nm (Fig. 14) (H1).

The small-diameter ion exchange resin that was used (Aminex A-7, $10 \pm 2\,\mu$) necessitated relatively high operating pressures; however, the use of a glass column necessitated a pressure limitation of 1000 psi or less. This resulted in an operating time of as much as 65 hr for a single urine analysis.

In Hamilton's early work, the sample was placed on the ion-exchange resin by removal of liquid at the top of the column and injection of the sample directly onto the top of the resin bed while the eluent flow was stopped. This is an adequate means of sample introduction, although an automated system can probably also be used. The chromatogram was developed with the stepwise elution by sodium citrate buffers of varying concentrations and pH from a typical sample of 0.5 ml of the body fluid (Fig. 15).

3.11. Organic Acid Analyzer

An organic acid analyzer for physiological fluids has not been developed to the same degree as the other systems. However, this type of analysis is of sufficient importance that it has been included in this pres-

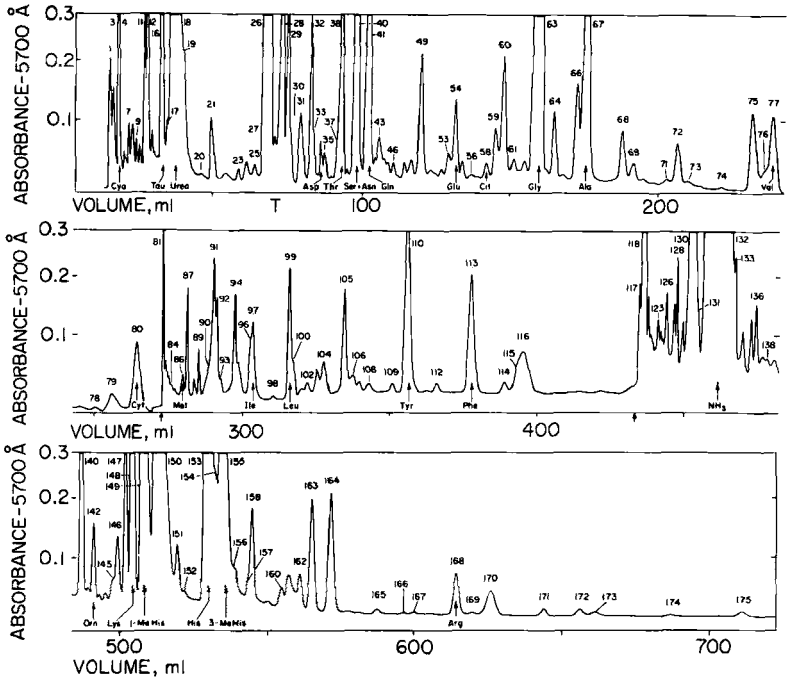


FIG. 15. High-resolution chromatogram of the ninhydrin-positive compounds in 0.5 ml of human urine. This was a single cation-exchange separation using step elution that required 65.5 hours. From Hamilton (H1, H3), *Handbook of Chemistry*, 2nd Ed., p. B-92, with permission.

entation. Several workers have attempted to use organic acid analyzers for determining organic acids in physiological fluids. Typical of these is the system used by Rosevear *et al.* (R1), which probably has the highest resolution and sensitivity reported for analysis of organic acids in physiologic fluids. Rosevear's system is an extensively modified version of a commercial instrument (Fig. 16). It uses a temperature-controlled glass chromatographic column (175 cm × 0.4 cm) operating at 20°C with eluent pressures up to 1000 psi; pulsating piston pumps for eluent delivery and colorimetric reagent metering; and a continuous colorimetric monitoring of the eluted organic acid by mixing and reacting an indicator reagent with the column eluate, followed by continuous detection with a flow colorimeter.

The separation medium is activated silicic acid with a particle size of 10–40 μ, which is packed into the column by a dynamic introduction of a slurry. Unfortunately, a new column must be packed for each analysis. This is a weak point in the system, and it is an obvious area

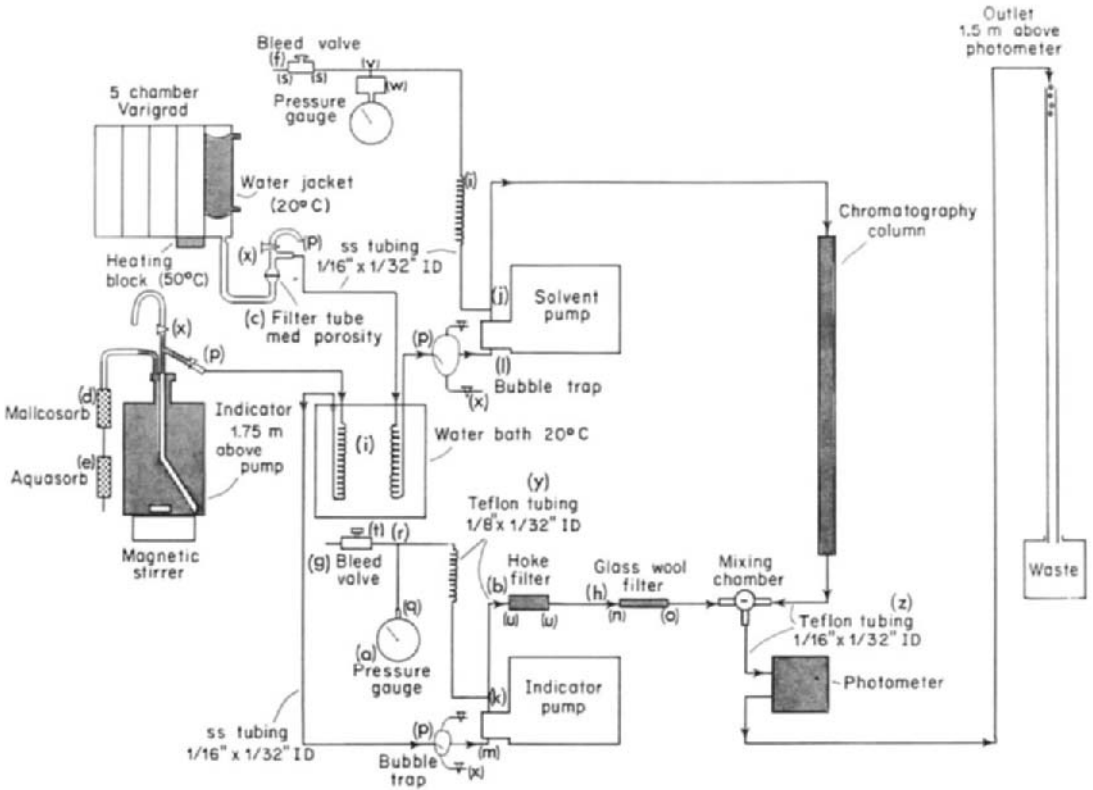


FIG. 16. Modified system for organic acid chromatography of body fluids. From Rosevear (R1), copyright © 1970 *Clinical Chemistry*.

for future development. It should be pointed out that a few years ago many liquid chromatographic separations were operated in a similar mode.

The eluent stream is a mixture of chloroform and *tert*-amyl alcohol with the addition of a small amount of water for adjusting the activation of the silicic acid. A multichamber gradient generation system was used to vary the eluent organic solvent makeup from essentially pure chloroform to a 1:1 mixture of the two solvents.

The colorimetric monitoring system used an ethanol solution of the indicator, neutral red (3-amino-7-dimethylamino-2-methylphenazine), that is mixed continuously with the column eluate and then monitored by a flow colorimeter at 550 nm.

Although the organic acid fraction of physiological fluid samples can be introduced into the system in several ways, one means is to presorb the sample on silicic acid, and then add this sorbent to the top of the column after which the gradient elution is started. This necessitates an additional manual operation that also presents a future area for development. A typical analysis requires 0.1 to 0.2 ml of the body fluid sample with an analysis time of about 6 hours (Fig. 17).

4. Experimental Results and Applications

High-resolution analyzers have been used to determine the molecular constituents of urine and blood serum as well as other body fluids, such as cerebrospinal fluid, perspiration, saliva, and amniotic fluid. Well over 300 molecular constituents can apparently be separated by a combination of all four types of analyzers; however, many of the separated components have not actually been isolated and identified by spectral and chemical tests.

4.1. Chromatographic Results

The UV-analyzer normally separates 100–120 chromatographic peaks from a urine sample in a 24-hour run (Fig. 9) (S5); however, as many as 140 peaks have been separated from a single urine sample, and over 180 different components were separated from urine that had been concentrated by a sorption process (M3). Sensitivity levels of less than a microgram are observed for many components (Fig. 10).

The carbohydrate analyzer has separated as many as 48 chromatographic peaks from a single body fluid sample; however, chromatograms from urine samples of normal subjects have 30–40 peaks (Fig. 13) (S5). The carbohydrate analyzer is sensitive to a few micrograms of each individual carbohydrate.

The common amino acids are well separated by the conventional amino

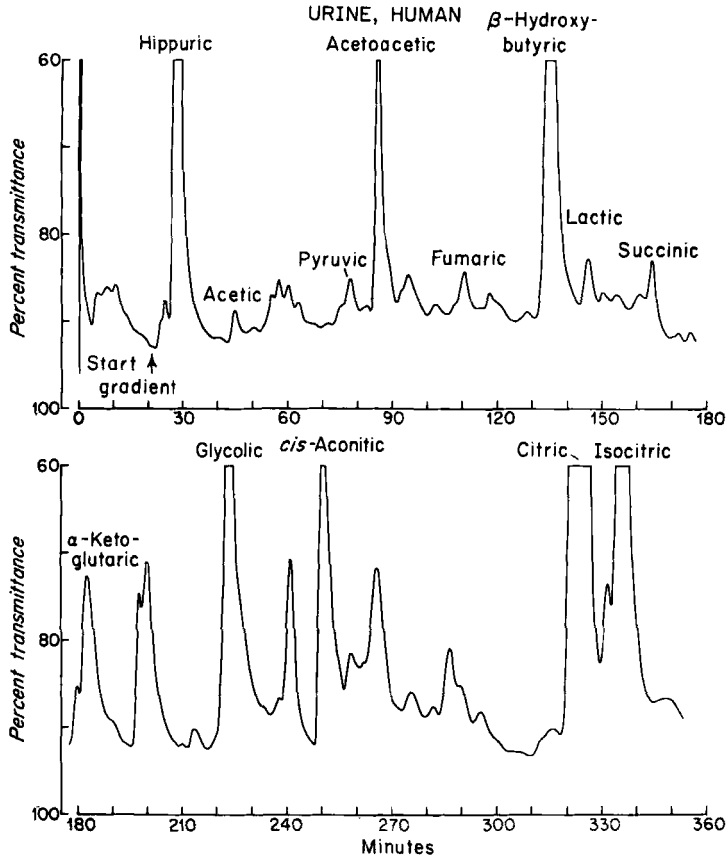


FIG. 17. Chromatogram of organic acids in 0.1 ml of human urine. From Rosevear (R1), copyright © 1970 *Clinical Chemistry*.

acid analyzer, particularly those analyzers using two-column systems. When such systems are adjusted for physiologic fluid analysis, they separate 30–50 ninhydrin-positive peaks from serum (E1) and about twice as many peaks from urine. In general, this requires an extensive increase in the analysis time. Although fewer analytical data are available on high-resolution analyses of the ninhydrin-positive components of body fluids using a single cation exchange column, at least 175 such components have been isolated from urine, with the indication that perhaps additional resolution would result in additional peaks (Fig. 15). Submicrogram sensitivity has been demonstrated (H3).

There is an indication that the organic acid analyzer can provide meaningful resolution of more than 50 constituents in urine (Fig. 17) (R1). Again, the sensitivity will be less than a microgram for some components.

In general, the components being separated and quantified by these analyzers are of relatively low molecular weight (less than a molecular weight of 1000). In fact, the high-molecular-weight components are usually removed by ultrafiltration or precipitation for the ninhydrin-positive compound analyzer and, in some cases, for the other analyzers. The detected compounds are thus the metabolic and catabolic products of the life processes.

Body fluids other than urine have considerably less complex low-molecular-weight component spectrums, at least at the concentration levels that can be detected by these analyzers. For example, blood serum samples, when compared with urine, will have about one-fourth as many chromatographic peaks of UV-absorbing constituents and carbohydrates and about one-half as many ninhydrin-positive and organic acid chromatographic peaks. Cerebrospinal fluid appears to have about the same complexity in UV-absorbing and carbohydrate components as does blood serum, and perspiration falls somewhere between urine and serum.

4.2. Identification of Separated Constituents

Actual identification of the separated body fluid constituents requires major experimental effort. Chromatographic peaks can be tentatively identified by comparing their chromatographic properties with those of reference compounds. However, confirmation of the identification requires isolation of the column eluate fraction represented by the chromatographic peak and determining the identity of the included constituent by chemical and spectral methods. The gas chromatograph and mass spectrometer have proved invaluable in this work.

So far, the tentative chromatographic method has been used to make most of the identifications of the ninhydrin-positive and organic acid components, especially for urine constituents. This simply requires that the unknown peak has the same elution volume as a known reference compound. A significant effort has been made to provide more definite identifications for the components separated by the UV- and carbohydrate analyzers. To date, this has included over 70 UV-absorbing compounds and 18 carbohydrates, some of which are listed in Tables 1–3 (B2, M2). Tentative identification of many more compounds has been made in all four systems, and, hopefully, the efforts in confirmative identification will continue.

TABLE 1

SOME OF THE COMPOUNDS SEPARATED FROM THE URINE OF NORMAL SUBJECTS BY THE UV-ANALYZER AND IDENTIFIED BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY[a]

Compound	UV λ_{max} (nm)	MU value for TMS derivative[j]	Mass spectral data[b]				
			Base peak	*m/e* (2)	*m/e* (3)	*m/e* (4)	Mol. wt.
Urea[c]	—[d]	12.44	44	60	17	43	60
Creatinine[c]	232	15.57[e]	42	43	113	112	113
β-Pseudouridine[c]	262	23.68	208	141	125	165	244
Uracil[c]	261	13.30	112	42	68	69	112
5-Acetylamino-6-amino-3-methyluracil[c]	263	—[f]	156	198	71	155	198
N^1-Methyl-2-pyridone 5-carboxamide	258	18.65	152	136	108	135	152
7-Methylxanthine[c]	269	20.19	166	68	123	67	166
3,7-Dimethylxanthine[c]	273	—[f]	180	67	109	82	180
Hypoxanthine[c]	249	17.92	136	81	109	108	136
Xanthine[c]	267	20.05	152	109	81	54	152
3-Methylxanthine[c]	269	19.26	166	68	95	123	166
1-Methylxanthine[c]	267	20.37	166	109	81	137	166
Uric acid[c]	276	21.22	125	69	168	97	168

2-Amino-3-hydroxybenzoylglycine	258	—[f]	136	121	224[g]	210	210
Phenylacetylglutamine[c]	258	23.50[e]	91	187	142	278[g]	264
4-Acetylaminobenzoylglycine[h]	267	—[f]	120	162	250[g]	208	236
Hippuric acid[c]	224	18.05	105	77	135	134	179
Citric acid[c]	—[d]	18.50	—[i]	—	—	—	192
3-Methoxy-4-hydroxybenzoylglycine	254	23.37	151	225	123	239[g]	225
3-Methoxy-4-glucuronosidobenzoic acid[h]	263	—[f]	168	151	153	358[g]	344
3-Methoxy-4-hydroxyphenylacetic acid[c]	279	17.61	137	182	122	92	182
4-Acetylaminobenzoic acid[h]	266	18.39	137	120	179	108	179
4-Hydroxyhippuric acid	253	22.10	121	195	150	93	195
3-Ethoxy-4-hydroxybenzoylglycine[h]	253	—[f]	137	165	107	239	239
3-Hydroxyhippuric acid	290	21.31	121	93	151	150	195
3-Ethoxy-4-glucuronosidobenzoic acid[h]	264	—[f]	154	137	182	165	358
3-Methoxy-4-hydroxybenzoic acid[c,h]	256	17.56	168	153	97	125	168

[a] From Mrochek *et al.* (M2).

[b] Includes base peak and three next most significant *m/e*.

[c] Reference compound available; data identical.

[d] Non-UV absorbing.

[e] MU value is for larger of two GC peaks.

[f] Multiple-GC peaks indicate decomposition.

[g] Methyl ester.

[h] Subject on artificial diet.

[i] Insufficiently volatile for MS; identified as TMS derivative with an integrated gas chromatograph–mass spectrometer.

[j] Methylene unit values from 6 ft × 0.25 in. OD glass column packed with 3% GC-SE-30 on 100/200 mesh Gas Chrom Q programmed from 100° to 325°C at 10°C/min.

TABLE 2

SOME OF THE COMPOUNDS SEPARATED FROM THE URINE OF SUBJECTS WITH VARIOUS PATHOLOGIES BY THE UV-ANALYZER AND IDENTIFIED BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY[a]

Compound	UV λ_{max} (nm)	MU value for TMS derivative[b]	Mass spectral data[c]				
			Base peak	*m/e* (2)	*m/e* (3)	*m/e* (4)	Mol. wt.
Trigonelline[d,e]	264	—[f]	138[g]	94	139	95	137
Nicotinamide-N-oxide[d,e]	268	17.21	122	106	78	138	138
Nicotinamide[d]	262	14.55	122	106	78	94	122
1,7-Dimethylxanthine	263	—[f]	180	68	123	95	180
Allopurinol[d]	249	16.12	136	73	135	109	136
Oxipurinol[c]	253	18.43	109	152	52	53	152
3-Methoxy-4-hydroxyacetanilide	244	18.92	139	181	124	96	181
4-Hydroxyacetanilide	242	17.92	109	151	81	95	151
Orotidine[c,d]	266	—[f]	—[h]	—	—	—	288
3-Methoxy-4-glucuronosidoacetanilide	242	—[f,i]	—[h,i]	—	—	—	357
4-Glucuronosidoacetanilide	240	—[f,i]	—[h,i]	—	—	—	327
Sulfanilamide[d]	263	21.53	172	156	92	108	172
Orotic acid[d]	276	17.50	68	156	113	69	156

3-Methoxy-4-hydroxymandelic acid	279	18.85	153	137	93	198	198
3-Methoxy-4-hydroxyphenyllactic acid	272	20.25	124	212	109	137	212
Phenyllactic acid[d]	259	15.85	91	148	103	166	166
4-Hydroxyphenylacetic acid[d]	278	16.28	107	166[g]	152	77	152
Benzoic acid[d]	272	12.30	122	105	77	51	122
2-Hydroxybenzoic acid[d]	302	15.14	120	92	138	64	138
2-Hydroxyhippuric acid[d]	300	20.88	121	120	62	92	195
4-Hydroxybenzoic acid	250	16.28	121	138	93	65	138
2,5-Dihydroxyphenylacetic acid[d]	295	18.40	122	94	150	182[j]	168
2-Hydroxyphenylacetic acid[d]	274	15.75	134	106	107	78	152

[a] From Mrochek *et al.* (M2).

[b] Methylene unit values from 6 ft × 0.25 in. OD glass column packed with 3% GC-SE-30 on 100/200 mesh Gas Chrom Q programmed from 100° to 325°C at 10°C/min.

[c] Includes base peak and three next most significant *m/e*.

[d] Reference compound available; data identical.

[e] Compound not previously reported in human urine.

[f] Multiple-GC peaks indicate decomposition.

[g] Mass spectral data identical to standard recovered from buffer used in anion-exchange separation.

[h] Insufficiently volatile for mass spectrometry.

[i] Hydrolyzed sample gave same data as parent compound.

[j] Methyl ester.

TABLE 3
SOME OF THE COMPOUNDS SEPARATED FROM URINE OF NORMAL SUBJECTS BY THE CARBOHYDRATE ANALYZER AND IDENTIFIED BY GAS CHROMATOGRAPHY[a]

Carbohydrate	Source	MU value[b]		
		Peak 1	Peak 2	Peak 3
Sucrose	Reference	27.46	—	—
	Urinary	27.42	—	—
Lactose	Reference	27.34	28.60	—
	Urinary	27.31	28.58	—
Allulose	Reference	18.61	18.83	19.43
	Urinary	18.57	18.80	19.41
Fructose	Reference	18.60	18.71	—
	Urinary	18.57	18.71	—
Arabinose	Reference	16.47	16.78	17.10
	Urinary	16.45	16.75	17.07
Fucose	Reference	16.71	17.06	17.46
	Urinary	16.75	17.07	17.48
Galactose	Reference	18.68	19.11	19.53
	Urinary	—	19.08	19.55
Sorbose	Reference	18.41	19.09	20.00
	Urinary	—	19.16	20.00
Xylose	Reference	17.38	17.94	—
	Urinary	17.44	18.00	—
Glucose	Reference	18.89	19.35	20.31
	Urinary	—	19.31	20.25

[a] From Mrochek *et al.* (M2).

[b] Methylene unit values from the single or multiple peaks that result from the GC separation on 180 cm × 6.3 mm OD glass column packed with 5% SE-30 on 100/200 mesh Chromosorb W(HP) programmed from 100° to 325°C at 10°C/min.

4.3. NORMAL VALUES

For high-resolution techniques to have general utility, it must be established that the body fluids of normal subjects have a definable normal spectrum of chemical constituents and that various pathologic states can be associated with abnormal values of one or more of the constituents.

UV and carbohydrate chromatograms from urine (24-hour composites) and serum samples from clinically normal subjects are very similar. About three-fourths of the major peaks are common to all the normal subjects tested, and the concentrations (peak sizes) are within relatively narrow limits (e.g., see Fig. 18) (J2). Variation during the diurnal cycle is measurable but not prohibitive (Fig. 19), and variations during long periods of time are much less for one person than the variation from person to person (J2).

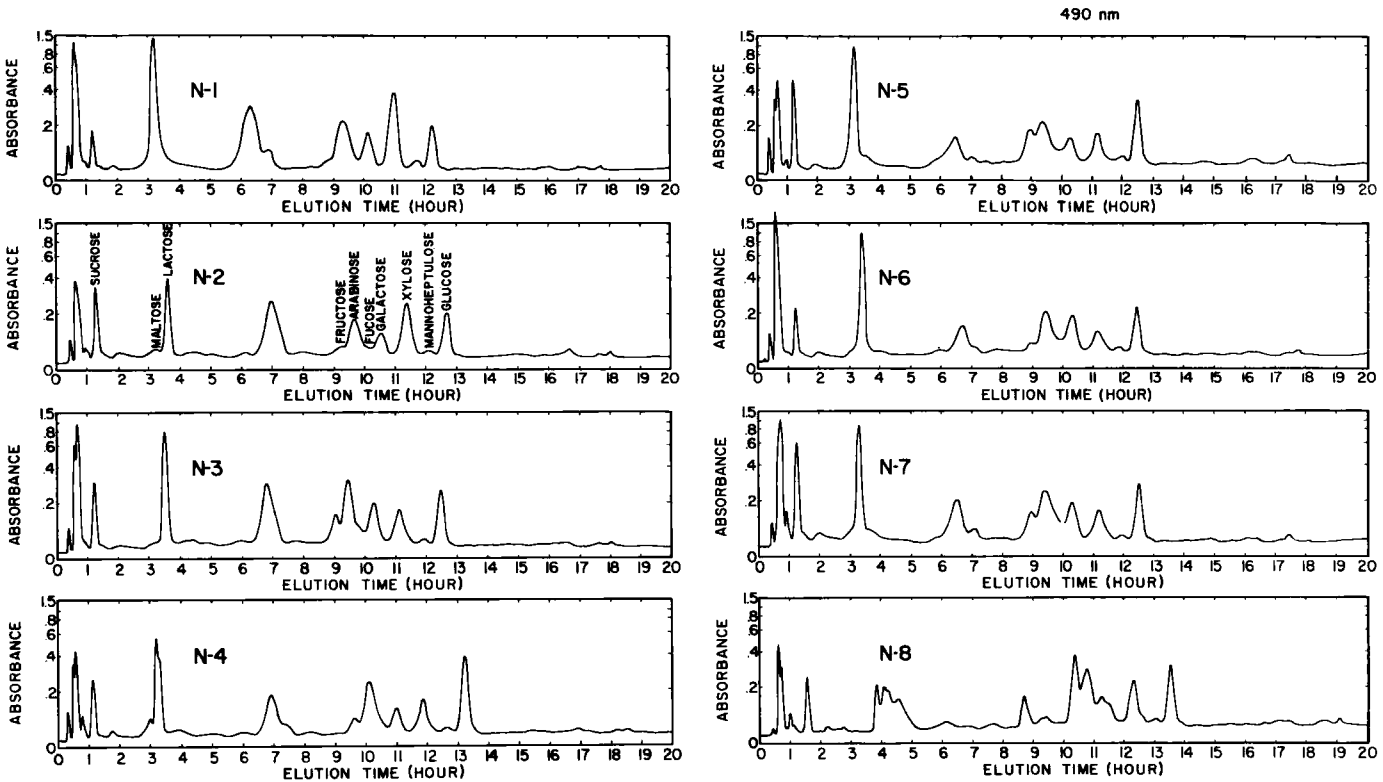


FIG. 18. Typical urine carbohydrate chromatograms of eight normal subjects on random diets. From Jolley *et al.* (J3) with permission.

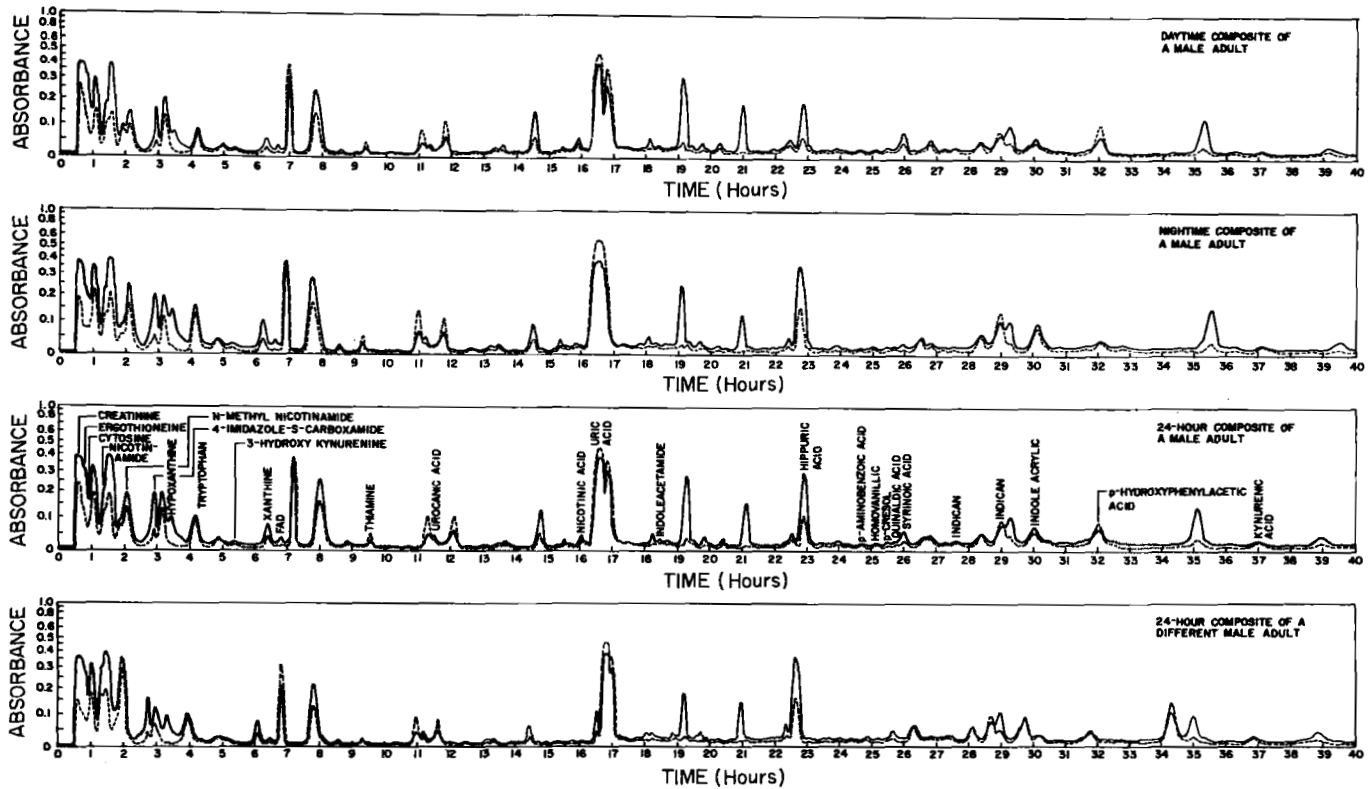


FIG. 19. UV chromatograms of the urine of normal subjects showing the effect of the diurnal cycle and the comparison between normal subjects. Run conditions: column, 0.45 cm ID × 200 cm 316 stainless steel with 10-μ diameter Bio-Rad AG1-X8; urine samples, 2 ml each; temperature, 25°C increasing to 60°C after 15 hours; pressure, 1500–2300 psig; elution, sodium acetate–acetic acid buffer at a pH of 4.4, varying in concentration from 0.015 M to 6 M at an average flow rate of 28 ml/hour. (—, 260 nm; — — — —, 280 nm.) From Scott (S12), copyright © 1968 *Clinical Chemistry*.

Normal values can be altered by dietary factors, especially when unusual diets are used (e.g., synthetic diets such as Vivonex) (Y2), and by the ingestion of drugs (K3). However, these effects can be predictable, and nominal control over food and drug intake is sufficient to allow establishment of base-line chromatograms. Normal subjects on identical diets produce chromatographic patterns that are almost superimposable.

4.4. Differences in Pathological States and During Drug Intake

Significant differences have been noted between normal and pathological urine chromatograms. For example, the urine chromatogram of a patient with a neuroblastoma had very large homovanillic acid and vanillic acid peaks, indicating that these excretion products may be useful indicators of that pathological state (J3). The lack of hippuric acid was noted in the urine of a patient with the Lesch-Nyhan syndrome (Fig. 20) (J3). This, coupled with an increase in benzoic acid excretion, indicated that the glycine conjugation mechanism may have been impaired in that pathological state.

These two examples show the utility of the "spectral approach" or establishment of the "chemical profile" of the body fluids that can now be achieved with high-resolution analyzers. Chemical indicators of abnormal states can be found without prior knowledge of their existence and, thus, without having to decide which specific chemical indicators are to be investigated.

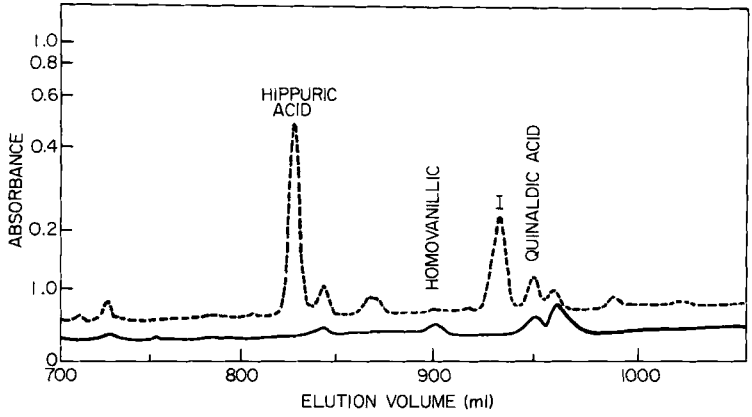


Fig. 20. Comparison of a selected portion of the UV chromatograms of a normal reference urine (— — —; *A*, 260 nm) and urine from an individual with the Lesch-Nyhan syndrome (—; *A*, 260 nm). From Jolley (J3), copyright © 1970 *Clinical Chemistry*.

Allopurinol, an analog of hypoxanthine, is widely used in the treatment of hyperuricemia and gout. The drug is a potent inhibitor of xathine oxidase, which is the enzyme catalyzing formation of uric acid, and thus it decreases endogenous synthesis of this purine. Researchers using the UV-analyzer found that the drug and its metabolite had a previously unknown side effect, namely, the inhibition of endogenous pyrimidine synthesis (K3). This resulted in a large increase in orotic acid and orotidine excretion and a corresponding decrease in uridine and other purines which may have contributed to undesirable side effects.

The carbohydrate analyzer has shown that there are considerable differences in excretion patterns of carbohydrates in disease. Many carbohydrates are excreted in excess in renal glycosuria and diabetes mellitus (Y1). Other abnormalities, such as pancreatic insufficiency and lactose deficiency, show several carbohydrate excretion abnormalities. The presence of large amounts of xylulose and other sugars during ingestion of xylose indicates that the xylose tolerance test may not be a true measurement of absorption since that sugar apparently also metabolizes (Y1).

Many other useful results have been found with high-resolution systems, and many of these have been reported in the previously mentioned symposia series (S4, S6, S8).

5. Utility and Future of High-Resolution Analytical Systems

What would one expect to gain from being able to analyze body fluids for their molecular constituents and, thus, obtain the chemical spectrum of the body? Obviously, in a more restricted sense this same question must be answered when any clinical laboratory test is being considered. If a single chemical constituent is being evaluated, its direct biochemical relationship may indicate the malfunction of a vital organ, the deficiency of an enzymatic system, the effect of a drug or other therapy, hormonal imbalance, etc. There are also pathologies where analyses for more than one constituent will allow a much better differentiation of a specific abnormality. High-resolution analytical techniques will be useful in both cases; and since a much larger number of variables will be measured, many abnormalities can be considered at the same time.

Conceivably then, the vast amount of data available from such analyses would give the clinician an additional, extremely useful tool. This will probably be the case sometime in the future; but, as the systems are being developed, there are some very real problems associated with their actual utility.

5.1. Data Processing

The very fact that so many data are available makes it much more difficult for the clinician to use the data since he would have to have the time and background to properly evaluate this vast amount of additional information. It would not be useful to simply give the clinician the resulting graphical chromatogram and expect him to identify and quantify the results. It would also be very time consuming to manually quantitate all of the chromatographic data by graphical separation of the resulting chromatographic peaks, finding their areas, and equating them to the concentration of each separated component. Obviously, the data can be presented in a much simpler form with the addition of computerized data reduction.

The advent of relatively inexpensive, small, digital computers has made it possible to automatically evaluate the chromatographic data on-line as they are formed and end up with a tabulation of the quantity of constituents at the end of the run (C1, S7). It would also be a relatively simple matter to direct the attention of the clinician to those components that are outside normal limits.

5.2. Clinical Significance

At the present time, the clinical significance of an additional 300 or more body fluid constituents is not fully known and, thus, not totally useful to the clinician, although many of these components have been investigated in a rather restricted research mode. This problem will improve as additional analytical systems come into general use and additional pathological states are investigated. As indicated in a preceding portion of this paper, several interesting and useful findings have resulted from use of high-resolution systems, and these undoubtedly will continue.

5.3. Economics of High-Resolution Analyses

Will high-resolution analytical systems ever be economically feasible for large-scale use or will the cost and analysis time always be too excessive? These are important factors since analysis time for some systems may be as long as 65 hours and the cost may be as high at $100 per sample. Although these conditions may be acceptable in the research laboratory, they could not be used on a routine basis in the clinical laboratory. Here again much progress has already been made. For example, the first prototype model of the UV-analyzer had an analysis time of about 40 hours, but recent work has decreased that time to 16 hours (S9).

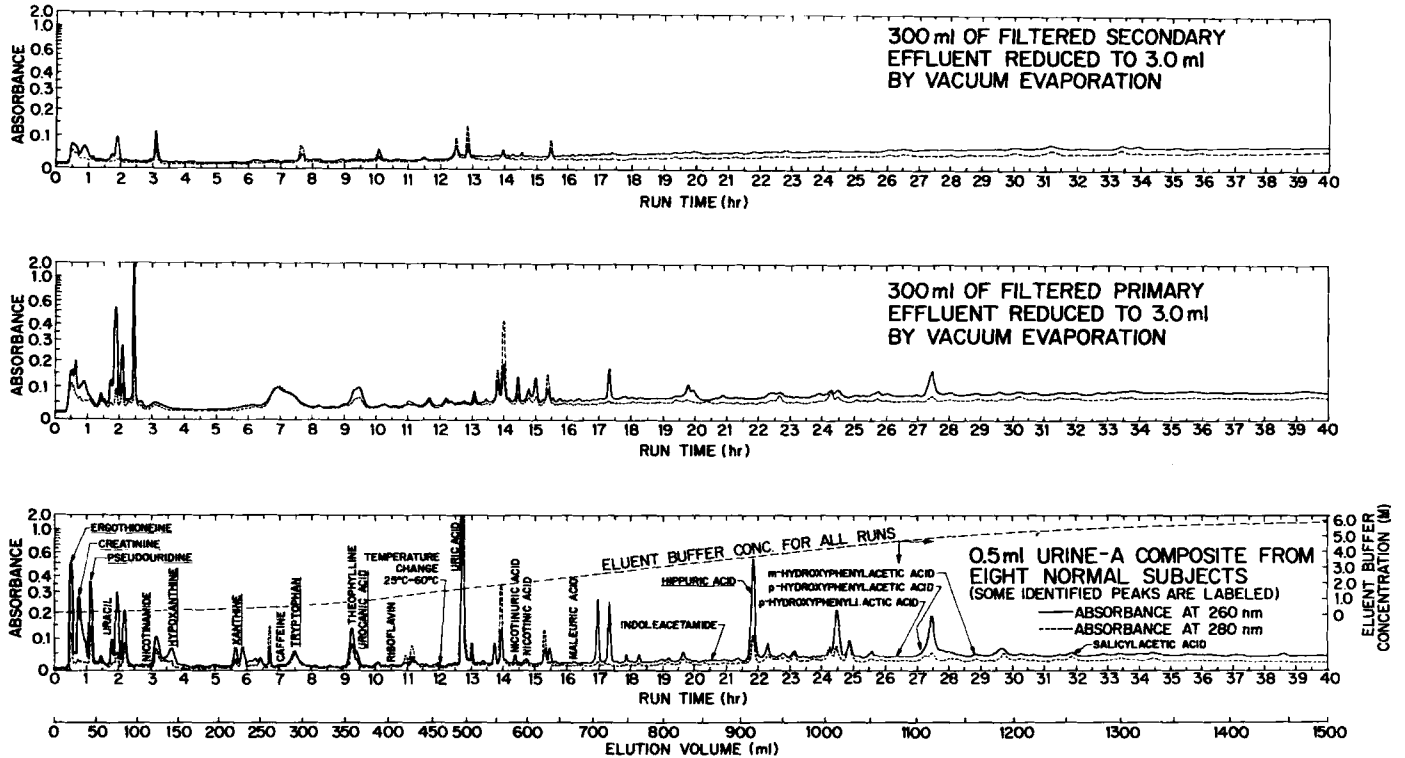


FIG. 21. Comparison between some of the molecular constituents of human urine and the effluent streams of the primary and secondary stages of a typical municipal sewage plant as determined by the Mark II UV-analyzer.

Another means of economy is the analysis of multiple samples with parallel columns in a single analyzer to given an additional sample throughput of at least eight times greater than at present (P2).

Additional development work will make the economic picture even more favorable. It should be remembered that a few short years ago it took over a day to perform a semiautomated amino acid analysis on a protein hydrolyzate, whereas it can now be performed in a highly automated way in less than 2 hours (B1). We may even reach the point where it will be less expensive, faster, and more accurate to make a high-resolution analysis of a body fluid sample even when we are interested only in a few of the constituents. This has certainly been true for the somewhat analogous case of trace metal analysis by the newer spectrographic methods instead of the more specific, but now less acceptable, wet chemistry methods.

5.4. Screening Laboratories

Finally, it is apparent that the field of medicine is moving toward the acceptance of and active pursuance of preventive medicine. The use of multiphasic screening laboratories is becoming more widespread to achieve this end. In such programs, the aim is to detect incipient diseases so they can be treated prior to the need for expensive hospitalization. So far, many of the tests and techniques used in these facilities have been adapted from the clinical laboratory that operates in the hospital where acutely sick patients are present. It is obvious that as we gain more knowledge of the chemical indicators of disease, tests that are more definitive for these indicators must be developed. High-resolution systems seem uniquely suitable to such a task as they become truly economic systems.

5.5. Other Uses

Uses of high-resolution analytical systems in other types of research can also be envisioned. For example, the molecular pollutants, especially the refractory organic compounds, in the effluents of sanitary sewage plants have not been well established. Preliminary results from analysis of primary and secondary effluents from conventional sanitary sewage plants show that up to 80 UV-absorbing constituents can be monitored by the UV-analyzer (Fig. 21). Obviously, such analytical systems would be useful in monitoring the effectiveness of various processing steps.

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ACID PHOSPHATASE[1]

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1. Introduction

The existence of the enzyme acid phosphatase was first revealed in 1925 when Demuth (D11) observed that human urine was capable of hydrolyzing hexose diphosphate with optimal activity occurring at a pH of approximately 5.0. In 1935 and 1936, Kutscher and his associates (K12, K13) found that this enzyme was present to some extent in the human testis, epididymis, seminal vesicle, spermatic cord and, in a remarkably high concentration, in the prostate. Within the next few years, Gutman and his associates (G10, G11, G12, G13, G14) determined that this enzyme activity was present in serum and could be utilized as an indicator of the presence of carcinoma of the prostate.

Since these early discoveries, this enzyme has assumed additional biological and medical significance. In 1955, de Duve and his associates described the association of acid phosphatase with "a special class of cytoplasmic granule" in rat liver (A13, D10), and this enzyme subsequently became the marker for a new intracellular component, the lysosome. Recently, lysosomal acid phosphatase deficiency has been described in man (N1). As the study of acid phosphatase progressed, increasing indications arose that there might be differences among the acid phosphatases of different tissues (A4, D13, K3), and more recently, the activities of acid phosphatases in platelets (Z1), in normal and abnormal leukocytes (B8, L7, L8, V1), and in Gaucher cells (L8) have been described to be indicators of corresponding pathological states. In addition, it has been shown that the acid phosphatase within a given tissue may

consist of more than one molecular form, or isoenzyme (L14, L15, S24, S31), and this finding has had genetic (H11) and pathophysiological implications. And even with regards to the earliest utilization of acid phosphatase, its determination in the serum as an indicator of the presence of carcinoma of the prostate, methodological advances and increasing clinical biochemical correlations have tended to define this role more precisely.

In view of these various considerations, it seemed most appropriate to review the various aspects of this subject for readers of these Advances, particularly since no review has appeared since the inception of the series in 1958.

2. Methods of Determination of Acid Phosphatase Activity

2.1. Introduction

A considerable number of procedures have been utilized to assay the acid phosphatase activity of serum, blood cells, and tissues. These have involved different substrates or concentrations of substrates, different temperatures, buffers, or variations in other conditions. If the same acid phosphatase were being measured, then the results were naturally not comparable. But the possibility also exists that closely related but different acid phosphatases were present within the same tissue or in different tissues, and the rate of action of these acid phosphatases depended on the particular substrates, buffers that were employed, or other conditions of the reaction. It seems most appropriate then to preface our review and consideration of the literature by describing briefly the conditions characterizing the most frequently used procedures for the determination of acid phosphatase activity, particularly in the serum. Other methods, or modifications of those to be presented here, will be described in later sections of this review.

2.2. The Gutman Method (G10, G14)

This was the first method used in assaying serum acid phosphatase activity and was a modification of the King-Armstrong (K4) method for alkaline phosphatase. The buffer-substrate was 0.005 *M* disodium monophenyl phosphate in Sörensen's 0.1 *M* citrate buffer adjusted to pH 4.8. To 10 ml of this mixture, brought to 37°C, 0.5 ml of serum at 37°C was added, yielding a pH of 4.9. The contents were stirred and allowed to incubate for 3 hours; the liberated phenol was determined. The activity was defined in units, as the number of milligrams of phenol liberated in 1 hour at 37°C by 100 ml of serum. For serums of high activity, shorter times of incubation or dilutions of serum were used. The normal range

was stated as lying between 0 and 2.5 units, but this will be considered more fully later (Section 6.2).

Shortly after publication, this procedure was utilized by several investigators with only minor changes, such as in the pH of the citrate buffer (H7, W1) the use of a sodium acetate–acetic acid buffer at pH 5.0 (H17) or, subsequently, the determination of hydrolyzed phenol with aminoantipyrine (K4). The concentration of phenyl phosphate, before the addition of serum, was 0.005 *M* in all these instances. Since the innovation of Gutman and Gutman (G10, G14) consisted in adapting the use of the phenyl phosphate in the King-Armstrong method for alkaline phosphatase to the determination of acid phosphatase at pH 5.0, the procedure will be referred to in this paper as the Gutman method.

2.3. The Bodansky Method (B18, J2)

This procedure was based on the use of β-glycerophosphate as substrate as in A. Bodansky's method for alkaline phosphatase (B17). In this method the mixture, to which the serum was added to start the reaction, had concentrations of 0.016 *M* sodium β-glycerophosphate and 0.021 *M* sodium diethylbarbiturate. The addition of 1.0 ml serum to 10 ml of this mixture or, as in a later version, of 0.5 ml to 4.5 ml of the mixture led to concentrations in the final reaction mixture of 0.0144 *M* sodium β-glycerophosphate and 0.019 barbiturate buffer.

In the first brief description of his procedure for acid phosphatase, A. Bodansky incorporated acetic acid in the β-glycerophosphate-diethylbarbiturate mixture so as to bring it to pH 5.0 before adding serum (1 volume to 9 volumes of mixture) to start the reaction. This proceeded for a period of 3 hours at 37°C, when it was terminated by the addition of trichloroacetic acid. The acid phosphatase activity was expressed as the number of milligrams of inorganic phosphorus liberated as phosphate in 1 hour by 100 ml of serum under the conditions of this assay. The normal range of acid phosphatase values was defined as lying between 0.1 and 0.4 unit (J2). In a later version (B18), the diethylbarbiturate buffer was omitted; to 6.93 ml of unbuffered 0.016 *M* sodium β-glycerophosphate, 0.27 ml of 0.50 *N* hydrochloric acid was added. The mixture was warmed to 37.5°C, and 0.8 ml of serum also warmed to 37.5°C was added. The mixture was allowed to incubate for 2 hours, when the reaction was stopped by the addition of trichloroacetic acid. The acid phosphatase activity was expressed in units as before. The average activity in 43 males was 0.19 unit with a standard deviation of 0.048 unit (B19). It is of interest in this connection that, in the course of studying a patient with prostatic carcinoma and extensive prostatic calcification, Barringer

and Woodard (B5) determined the action of serum on β-glycerophosphate over a range of pH levels from 6.0 to 9.0 and found the ratio of activity at pH 6.4 to that of 9.0 elevated in some cases of prostatic carcinoma. However, no definite method for serum acid phosphatase activity was proposed at this time.

Shinowara *et al.* (S18) made a slight modification in the Bodansky procedure. To 50 ml of stock substrate solution containing 0.0317 *M* sodium β-glycerophosphate and 0.0412 *M* sodium diethylbarbiturate, 5 ml of 1.0 *M* acetic acid was added, and the solution made up to 100 ml. The pH was 5.0 or was adjusted to this pH. One volume of serum, undiluted or diluted to various strengths, was added to 10 volumes of the diluted substrate, and the reaction was allowed to proceed for 1 hour when it was stopped by the addition of trichloroacetic acid. The concentrations in the reaction mixture, which had a pH of 5.0, were 0.0144 *M* sodium β-glycerophosphate, 0.0185 *M* diethylbarbiturate, and 0.045 *M* acetate. The units were expressed as in the Bodansky method, namely, milligrams of phosphorus liberated per 100 ml of serum in 1 hour, and the range was 0.0–1.1 units in 20 healthy subjects and in 140 control patients.

2.4. The *p*-Nitrophenyl Phosphate Method

The relative rates of hydrolysis of various phosphate esters, including *p*-nitrophenyl phosphate, at alkaline pH levels ranging from 8.08 to 9.80, were studied by King and Delory in 1939 (K5a). In 1937 Ohmori (O1) had investigated the hydrolysis of *p*-nitrophenyl phosphate at pH levels ranging from 2.0 to 9.0 by various "phosphatase" preparations from pig kidney, dried yeast, guinea pig blood, and "takaphosphatase." He noticed that several of these preparations showed optimal activities in the acid region, at about pH 4.0–5.0.

In 1947, Hudson *et al.* (H15) developed a method for acid phosphatase which, like the procedure of Bessey *et al.* for alkaline phosphatase (B16), was based upon the use of *p*-nitrophenyl phosphate as substrate. The buffer substrate solution consisted of equal volumes of a 0.1 *M* sodium acetate–acetic acid buffer, pH 5.4, and 0.001 *M* magnesium chloride and of a 0.4% solution of approximately 50% pure disodium *p*-nitrophenyl phosphate in 0.001 *N* HCl. To 1 ml of this solution, 0.1 ml of the serum sample was added. The final concentrations in this reaction mixture were 0.045 *M* acetate buffer, pH 5.4; magnesium chloride. 0.00045 *M*; substrate, 0.004 *M*. The reaction was allowed to run for 30 minutes at 38°C, and the reaction was stopped by the addition of sodium hydroxide. The liberated yellow *p*-nitrophenol was read at 400 nm and the amount was

calculated from a suitable calibration curve. The units of acid phosphatase activity were defined as the number of millimoles which were liberated in 1 hour by 1 liter of serum. In 47 normal individuals, the mean value was 1.54 units with a standard deviation of 0.34 unit.

2.5. Method of Huggins and Talalay (H18)

The principle of this method consists in the action of phosphatase, whether alkaline or acid, on phenolphthalein diphosphate to liberate phenolphthalein which, at alkaline pH, is pink or red. The intensity of the color was measured immediately, and the amount of phenolphthalein was determined from a suitable calibration curve. Huggins and Talalay (H18) synthesized their substrate and obtained a preparation which they believed to be sufficiently pure for their purposes. The procedure for the determination of acid phosphatase activity was as follows. To 5 ml of a solution containing about 0.001 *M* sodium phenolphthalein diphosphate, dissolved in 0.1 *M* acetic acid–acetate buffer of pH 5.4 and warmed to 37°C, 0.5 ml of serum or of another of acid phosphatase, also warmed to 37°C, was added. The contents were mixed, and the mixture was incubated for precisely 1 hour at 37°C; 4.5 ml of an alkaline glycine buffer, pH 11.2, was then added to stop the reaction; the color was read immediately. The units were expressed as the number of milligrams of phenolphthalein liberated by 100 ml of serum under the stated conditions. The serum acid phosphatase levels for 41 normal males, aged 21 to 65, and 15 normal females, aged 21 to 50, gave an average value of 5.9 units with a range of 3 to 10 units. No difference between the sexes was observed.

2.6. β-Naphthyl Phosphate Method

In 1950 Seligman and his co-workers (S13) suggested the use of sodium β-naphthyl phosphate as a substrate for the determination of acid or alkaline phosphatase activity. For the former, 1 ml of 1:20 diluted serum was added to 5 ml of 0.4 m*M* sodium β-naphthyl phosphate in 0.1 *M* acetate buffer of pH 4.8, and the reaction was allowed to proceed for 2 hours at 37.5°C. The addition of 4 drops of 1 *M* sodium carbonate solution served to retard the reaction as well as to raise the pH to the optimal level for coupling with 1 ml of a solution of tetrazotized orthodianisidine. After 3 minutes, the protein was precipitated with trichloroacetic acid, the dye extracted with ethyl acetate, and the color density determined in the region of 540 nm. The unit of phosphatase activity was defined as that amount of enzyme which liberates the color equivalent of 10 ml of β-naphthol per hour at 37.5° in 1 hour. The serum acid phosphatase in a group of normal adults ranged from 0.7 to 1.6 units and averaged 1.0 unit per 100 ml of serum.

2.7. α-Naphthyl Phosphate Method

Babson *et al.* (B1, B2) introduced the use of this substrate with the suggestion that it was highly specific for the presence of prostatic acid phosphatase in the serum. The substrate-buffer mixture is a commercially designed tablet containing 0.67 mg of sodium α-naphthyl acid phosphate in a mixture of citrates designed to yield a pH of 5.2 in the reaction mixture. To this tablet, dissolved in 0.5 ml of water and warmed to 37°C, 0.2 ml of serum was added and the reaction was allowed to incubate at 37°C for precisely 30 minutes. The mixture was cooled at 15–20°C, and a tablet containing 0.4 mg of tetrazotized orthodianisidine in a stabilized form was added and crushed with a glass rod. The solution was diluted to 5.0, and the optical density of the resulting colored solution at 530 nm was read exactly 3 minutes after the addition of the color developer. Suitable controls are employed. The unit of acid phosphatase was defined as the amount of enzyme that will liberate 1 mg of α-naphthol per hour. The activities of serum acid phosphatase in 56 apparently normal, healthy young men ranged from 0.9 to 5.5 units per 100 ml of serum and yielded a mean value of 2.0 ± 0.7 units. The activities in 33 apparently normal women ranged from 0.5 to 2.6 units and gave a mean value of 1.5 ± 0.5 units. Babson *et al.* (B2) believed that this method was highly specific for the prostatic component of serum acid phosphatase. They determined the activities of mixtures of heated serum with prostatic acid phosphatase or erythrocytic acid phosphatase on the series of substrates used in various methods. The ratio of activities was designated as the relative specificity for prostatic acid phosphatase and had the following values: phenyl phosphate, 2.3; phenolphthalein phosphate, 0.9; *p*-nitrophenyl phosphate, 1.2; β-naphthyl phosphate, 1.9; β-glycerophosphate, 48; α-naphthyl phosphate, 98. Thus, it would appear that the α-naphthyl phosphate is the most specific procedure for the determination of the presence of prostatic acid phosphatase. However, it should be noted that the assumption underlying the work of Babson *et al.* (B2) is that there are only two types of acid phosphatase in the serum, and it is quite possible that there are several others. In a study of 120 patients without cancer and 87 with prostatic cancer, Seal *et al.* (S12) found that the α-naphthyl phosphate substrate method was as sensitive or more sensitive than the tartrate-inhibitable phenyl phosphate substrate method.

2.8. Comparison of Acid Phosphatase Activities Determined by Different Methods

It is apparent from the preceding discussion that the rate of action of the acid phosphatase present in normal serum varies with the par-

ticular organic phosphate compound used as substrate and its concentration in the reaction mixture. Since the "acid phosphatase" in the serum is undoubtedly a mixture of the enzyme from various tissues, it would be irrelevant to carry out any precise kinetic studies at different concentrations of each substrate and thus determine Michaelis constants. The normal average values for the activities that have been noted above by the various methods may all be converted into micromoles of substrate hydrolyzed in 1 hour at 37–38°C by 100 ml of serum to yield the following comparison: β-glycerophosphate, 6.1; phenyl phosphate, 15; phenolphthalein diphosphate, 18; *p*-nitrophenyl phosphate, 145; β-naphthyl phosphate, 69; α-naphthyl phosphate, 14. As will be illustrated subsequently, the relative rates of action on the different substrates may differ even more widely in patients with elevated serum acid phosphatase activities arising by the admixture of acid phosphatases from different tissues.

As the preceding considerations illustrate and as was noted at the beginning of this section (2.1), comparison of acid phosphatase activities obtained in different studies must take into account the method employed. Some workers have attempted to do this by using the terms "β-glycerophosphatase," "phenylphosphatase," etc. to designate the substrate employed (B6, T6). However, such usage may imply that different "acid phosphatases" are responsible for these actions, and we shall therefore attempt to avoid this usage in the present review.

2.9. Current Methods for Determination of Serum Acid Phosphatase Activity

Most of the methods that are currently being employed in clinical laboratories or in investigations are either those that have just been described or are slight modifications thereof. For example, Linhardt and Walter (L9) have chosen for inclusion in Bergmeyer's "Methods of Enzymatic Analysis" (L9), the procedures of Huggins and Talalay (H18) utilizing phenolphthalein diphosphate as substrate and of Hudson *et al.* (H15) with *p*-nitrophenyl phosphate as substrate. The writer instituted the method of A. Bodansky (B18, J2) in the Department of Biochemistry, Memorial Hospital, New York in 1948, and this method is still being employed there. Levinson's and MacFate's text "Clinical Laboratory Diagnosis" (L6) has selected the method described by A. Bodansky (B18, J2) as modified by Shinowara *et al.* (S18). "Bray's Clinical Laboratory Method" (B7) has chosen the procedure of Hudson *et al.* (H15) except that citrate–citric acid buffer, pH 5.0, is substituted for the acetate–acetic acid buffer, pH 5.0. Automated methods based on

the manual methods that have been described above are also coming into use (G9, K9).

The question may arise as to which is the preferred method. In the author's experience, and this will be documented more completely later, the use of the substrate, sodium β-glycerophosphate, as in the Bodansky procedure (B18, J2), is more specific for elevations of serum acid phosphatase activity due to prostatic carcinoma. However, the use of other substrates, such as sodium phenyl phosphate in the Gutman method (G10, G14), may elicit alterations of activity in the serum that reflect diseases in other tissues.

2.10. Determination of Acid Phosphatase Activity in Blood Cells and in Tissues

As we have seen, practically all the methods on the determination of acid phosphatase activity in serum are calculated upon the amount of reaction product, such as inorganic phosphate, phenolphthalein, or *p*-nitrophenol, that would be produced under the conditions stated for the method by 100 ml of serum or, as in the method of Hudson *et al.* (H15), by 1 liter of serum. In the case of the acid phosphatase activity of tissues, some other basis for calculation is used, although the method may be the same as that used for serum.

A few examples will be cited here in illustration. In their study of the properties of acid phosphatases of erythrocytes and of the human prostate gland, Abul-Fadl and King (A4) employed a substrate–buffer mixture consisting of equal volumes of 0.02 *M* disodium phenyl phosphate and of acetate buffer (concentration not stated). The volume of hemolysate or of prostatic gland extract that was added to this mixture was not stated, and the reaction was allowed to proceed for exactly 30 minutes at 37°C. The activities were expressed as milligrams of phenol liberated per milliliter of red cells per hour or as milligrams of phosphate liberated in 30 minutes per 100 ml of enzyme solution. The red cell preparation was presumably a 1:10 hemolysate, but the precise dilution of the prostatic preparation was not given. Woodard (W6, W8) employed the method of A. Bodansky (B18, J2) in determining the acid phosphatase activity of various human tissues. She calculated her activities as the number of milligrams of phosphorus that would be liberated per hour by 1 g of tissue under the defined conditions of the assay. In assaying the distribution of acid phosphatase in the rat ventral prostate, Bertini and Brandes (B15) employed a total reaction volume of 0.40 ml containing 0.28 *M* sodium glycerophosphate (it was not stated whether this was α or β) in 0.05 *M* acetate buffer. Results were expressed as micro-

grams of liberated phosphorus after 10 minutes' incubation at 38°C, and calculated per gram of wet tissues. In studying the intracellular distribution of acid phosphatase in rat liver, de Duve and his associates (A12, A13, D9, D10, G2) measured the amount of inorganic phosphate liberated at 37°C in the presence of 0.05 *M* β-glycerophosphate and 0.05 *M* acetate, adjusted to pH 5.0, and expressed the activities as micrograms of P liberated in 10 minutes at 37°C per gram of liver. In studies of the acid phosphatase activity of leukocytes in normal persons and in patients with leukemia or other blood dyscrasias, the activities were expressed as milligrams of phosphorus liberated in 1 hour by 10^{10} cells from a reaction mixture at pH 5.0 containing a final concentration of 0.02 *M* sodium β-glycerophosphate as substrate (B8, B9, V1).

3. Acid Phosphatases from Different Tissues: Purification, Isoenzymes, and Properties

3.1. Introduction

Study of the distribution of acid phosphatase in different tissues is burdened by indications that there are several acid phosphatases. Even the older literature indicated the nonidentity of acid phosphatases of different origin. In 1934, Davies (D4) showed that the acid phosphatase in the red cell hydrolyzed α-glycerophosphate more readily than β-glycerophosphate, whereas the reverse was true for the acid phosphatase from spleen. Kutscher and Wolbergs (K12) found that prostatic acid phosphatase was inactivated irreversibly by various narcotics, including alcohols.

A more systematic study of the acid phosphatases of erythrocytes and of human prostate was undertaken in 1949 by Abul-Fadl and King (A4). The preparations were crude, the prostatic phosphatase being obtained by grinding human prostate with a 5-fold volume of 0.9% NaCl. The erythrocytic phosphatase consisted of centrifuged red cells, separated from white cells, washed twice with 0.9% NaCl and hemolyzed in 9 volumes of water. The buffer-substrate mixture consisted of equal volumes of acetate buffer (concentration not stated) and 0.02 *M* disodium phenyl phosphate.

The erythrocytic acid phosphatase from man and several other species showed two pH optima, one at a range of pH 4.3–4.8 and the second at pH 5.0–5.7. A concentration of 0.01 *M* Mg^{2+} inhibited these activities to the extent of about 30–50% at the lower pH levels and somewhat less so in the region of the higher pH optimum. Human prostatic acid phosphatase showed one clear pH optimum, at about 5.0–5.2, and the inhibition by 0.01 *M* Mg^{2+} was about 30% in this region.

The relative rates of hydrolysis of several substrates were determined at 37°C and pH 5.0 and expressed as milligrams of phosphorus liberated in 30 minutes per 100 ml of a diluted enzyme preparation. For 0.02 *M* β-glycerophosphate in the absence of any added Mg^{2+} these rates were 0.2 for erythrocytic phosphatase and 29 for prostatic phosphatase. The corresponding rates were 11 and 28 with 0.02 *M* α-glycerophosphate as substrate, and 55 and 53 with 0.005 *M* phenyl phosphate as substrate. The presence of Mg^{2+} activated the rates of hydrolysis to only a small degree. Thus, it may be seen that the use of β-glycerophosphate as substrate distinguished sharply between the erythrocytic and prostatic phosphatases.

Abul-Fadl and King (A1, A2, A3, A4) also investigated the effect of various ions and organic compounds on the acid phosphatase activity of these two tissues. Without describing the results in detail, some of the outstanding effects may be noted. A concentration of 0.5×10^{-3} *M* Cu^{2+} inhibited erythrocytic phosphatase to the extent of 88–96%, but prostatic phosphatase only to the extent of 10–18%. Similarly, 0.5% formaldehyde inhibited completely the erythrocytic phosphatase, but had no effect on prostatic phosphatase. The reverse patterns were shown by 0.5×10^{-3} *M* Fe^{3+} (ferric) ion, which inhibited erythrocytic phosphatase slightly, about 5–9%, and inhibited the prostatic enzyme to the extent of 80%. Fluoride in 0.01 *M* concentration also had comparatively little effect (8% inhibition) on erythrocytic phosphatase but exerted a marked inhibition, 96%, on prostatic phosphate. Of various organic radicals tested, only L-(+)-tartrate (0.01 *M*) had a marked differential effect, with 94% inhibition of the prostatic phosphatase and none of the erythrocytic phosphatase.

These results were among the first to indicate the diverse nature of acid phosphatases from different sources and were the forerunner of other studies designed to differentiate among the acid phosphatases from different tissues as well as procedures aiming to determine the tissue source of elevations of this enzyme in the serum.

An approximate idea of the distribution of acid phosphatase activity in human tissues, regardless of the nature of the acid phosphatase, may be obtained from the studies of Reis (R2) on 5′-nucleotidase and other phosphomonoesterases. He prepared aqeuous homogenates of postmortem tissue in the proportion of 20 parts of water to one of tissue, allowed these to autolyze for 2 days at room temperature, centrifuged the material, and employed the supernatant fluid. The assay mixture consisted of 0.4 ml of a suitable buffer, 0.1 ml of 0.005 *M* phenyl phosphate as substrate, and 0.1 ml of tissue extract. The enzyme activity was expressed as micrograms of phosphorus hydrolyzed per hour per milligram of wet

weight tissue. At pH 5.5, the following activities were obtained: thyroid, 0.22; testicle, 0.30; media of aorta wall, 0.9; brain cortex, 0.5; optic nerve, 0.3; pituitary posterior lobe, 0.6; pituitary anterior lobe, 0.7; liver, 0.3; lung, 0.4; kidney medulla, 0.9; kidney cortex, 1.9; ossifying cartilage, 0.2; duodenal mucosa, 0.1; jejeunal mucosa, 0.4; prostate, 1030.

The slight but definite elevations of serum acid phosphatase activity in conditions such as thrombocytopenia (O2, Z1), Gaucher's disease (T6, T8), or various myeloproliferative diseases (B6) indicate the possibility that platelets, the marrow, and the reticuloendothelial system may also be sources of acid phosphatase. These aspects will be discussed more fully later in the review.

3.2. Human Prostatic Acid Phosphatase

3.2.1. *Introduction*

The purification of acid phosphatase from the human prostate was undertaken, and high degrees of purity were obtained, before any solid information was available concerning the intracellular distribution of this enzyme or its existence in multiple molecular forms or isoenzymes. Accordingly, in this review several methods of purification will be described first, and the other aspects will then be considered.

3.2.2. *Purification of Human Prostatic Acid Phosphatase*

A few of the outstanding contributions in this area will be briefly described. London and Hudson (L10) began their purification by slicing frozen human prostates into slices 0.5–1 mm in thickness, and adding to these slices 3 g of 0.2 *M* acetate buffer, pH 5.0, for each gram of tissue. The mixture was allowed to extract for 48–72 hours in the refrigerator with occasional shaking and then strained through cheesecloth. The tissue residue was extracted twice more in the same manner; the three extractions yielded about 80% of the activity originally present in the prostatic tissue.

The combined extracts, which represented a 22-fold purification from the prostate, were dialyzed for 24 hours against distilled water at room temperature. The material inside the cellophane bag separated into a precipitate, which accounted for 60–70% of the total protein, and a clear supernatant containing over 85% of the activity and representing a 41-fold purification. This supernatant was then mixed with calcium phosphate gel at pH 7.5 and filtered. The filter cake was eluted with 0.02 *M* sodium citrate at pH 7.0, and the cake was washed with distilled water. The combined eluate, which showed an 81-fold purification, was concentrated by lyophilization. The enzyme solution was

adjusted to pH 5.8 with 0.2 *M* acetic acid cooled to 0°C and fractionated rapidly with acetone at 0°. The sediments from 36% acetone were discarded, and the sediments from 44% acetone were redissolved in half the starting volumes of distilled water. Treatment of this enzyme solution with ammonium sulfate between 60% and 68% saturation for 24 hours in the refrigerator resulted in a precipitate that represented 27% and a 296-fold purification of the enzyme activity in the original prostatic tissue. This material was dissolved in acetate buffer, diluted to 0.05% protein, placed in a gas washing bottle and caused to foam by passing in CO_2. The foam which contained almost all the protein was led off through the side arm. The remaining liquid or "frothate" was lyophilized and dialyzed against acetate buffers. Further concentration could be achieved by blowing hot air over the solution. The concentrated "frothate" amounted to 21% yield of the acid phosphatase originally present in the prostatic tissue and represented a 4900-fold purification. However, this preparation was unstable and even when kept in the refrigerator lost about 50% of its activity each month.

In 1958 Boman (B24) described a method of purification in two steps. The first one consisted of an extraction and a dialysis, and the second was a chromatographic fractionation. The starting material was human prostatic tissue and was stored at −15°C. The frozen prostatic tissue was cut into thin slices, weighed, and extracted with 5-fold its volume of 0.01% solution of Tween in cold distilled water at 4°C. After 24–48 hours, the pink opalescent extract was filtered through glass wool and dialyzed against distilled water for about 3 days. The precipitate formed during the dialysis was removed through centrifugation and discarded. The brownish pink supernatant was freeze dried. Of this material (fraction 1), 600 ml was dissolved in about 3 ml of McIlvaine's citrate phosphate buffer of pH 5.50 and was dialyzed against this buffer for 6–12 hours.

This solution was then applied to a column (72 × 3.3 cm) of Dowex 50 X-2 which had been equilibrated with the citrate phosphate buffer (B23). Elution was carried out successively with a citrate phosphate buffer of pH 5.0 and 6.00. About 50 fractions with a volume of 5–7 ml were collected. Two sharp peaks of protein concentration were obtained at about tubes 5–15 and tubes 27–29. The acid phosphatase activity was localized only in the second peak and represented a 10-fold purification.

A substantially different procedure for purification was employed by Ostrowski and his co-workers (O3, O4). The frozen human prostate gland was sliced into sections and weighed, and 5 volumes of 0.01% Tween 80 solution in distilled water was added. The mixture was homogenized in a Waring Blendor for 30 seconds at 13,000 rpm, stored in the cold

for 24 hr with occasional shaking, filtered through glass wool, and the residue was reextracted with 1 volume of distilled water. The combined filtrates were dialyzed against distilled water for 72 hours, with two changes of water. The pH was adjusted to 7.0 with ammonia, and the extract then centrifuged for 20 minutes at 3000 rpm. The clear, supernatant, pink solution, designated as FI, was poured off. It contained 2–5 mg protein per milliliter and, upon electrophoresis at pH 8.4, showed three separate peaks of protein a, b, and c migrating to the anode. Acid phosphatase as determined by hydrolysis of *p*-nitrophenyl phosphate was found only in fraction b, and diesterase activity, as determined by hydrolysis of bis(*p*-nitrophenyl) phosphate, was present between peaks b and c and within peak c.

The enzyme solution, FI, was brought to pH 7.0; powdered ammonium sulfate was added up to 45% saturation, and the solution was adjusted with ammonia to pH 7.0. The solution was cooled in the refrigerator for 24 hours, then centrifuged for 20 minutes at 3000 rpm, and the precipitate was discarded. To the supernatant ammonium sulfate was added up to 65% concentration and the pH readjusted to 7.0. After refrigeration as before, the precipitate was collected by centrifugation at 7000 rpm for 20 minutes.

The precipitate obtained by treatment with ammonium sulfate between 0.45 and 0.65 saturation was extracted with McIlvaine's buffer solution (0.077 *M* Na_2HPO_4–0.061 *M* citric acid) of pH 4.0 by stirring at 5° for 10 minutes. The mixture was then centrifuged at 10,000 rpm for 20 minutes and discarded. The supernatant, containing most of the acid phosphatase and relatively little of the protein of fraction 1 was clarified by centrifuging off sediment at 35,000 rpm for 60 minutes, then dialyzed successively against a large volume of distilled water for 24 hours, and against 0.0175 *M* sodium phosphate buffer of pH 7.0. This preparation, designated as FII, showed on electrophoresis three anodic protein peaks—a, b, c with b as the major peak. The entire acid phosphatase activity was present in the second peak, b; very little diesterase activity was evident, and it was confined to peak c.

The enzyme solution was then chromatographed on a DEAE-cellulose column. Elution with phosphate buffer and NaCl gave 5 peaks; the second and third peaks contained the acid phosphatase, with peak two showing a recovery being about 60% of the enzyme originally applied and containing most of the eluted enzyme. Diesterase was present chiefly in the fourth peak. The second peak (fraction FII-2) was rechromatographed on DEAE-cellulose and yielded a symmetrical and high activity peak, indicating a high degree of purification. This was designated at FIII. Starch gel electrophoresis at pH 8.5 showed a single sharply

defined band migrating to the anode; at pH 4.5 a single sharp band migrating slightly toward the cathode was similarly obtained. This preparation showed a sharp pH optimum at 4.8 with *p*-nitrophenyl phosphate as substrate. Although Ostrowski and Tsugita (O4) termed this preparation highly purified and homogeneous, they gave no value for the specific activity and hence for its degree of purification from the original prostatic tissue.

Davidson and Fishman (D3) submitted a relatively simplified method of purification. Sliced prostate was homogenized for 1 minute in a Waring Blendor with 1 volume of chipped ice and 3–4 volumes of ice-cold Tris·citrate buffer, pH 3.7. The homogenate was filtered in the cold, and the pH of the filtrate was adjusted by adding first an amount of 22% Tris buffer equal to 3% of the volume of the filtrate and enough dilute ammonia solution to achieve a pH of 7.5–8.5. The filtrate was chilled in ice and solid ammonium sulfate added and dissolved to attain 0.65 saturation. The resulting solution was centrifuged in the cold (0°) for 10 minutes. The resulting supernatant (No. 1) was discarded, and the precipitate was suspended in a volume of Tris·citrate buffer, pH 3.7, equivalent to no more than 10% of the volume of the original filtrate. Insoluble protein was removed by centrifugation, and the extract was treated with ammonium sulfate to attain 0.45 saturation. This supernatant (No. 2) was now active, but the precipitate could be extracted as before to yield another active supernatant (No. 3). In several preparations, the degree of purification of supernatant solutions 1 and 2, as judged by the specific activity, ranged from about 7- to 40-fold the activity of the original filtrate.

3.2.3. *Isoenzymes of Human Prostatic Acid Phosphatase*

The preceding description of the use of chromatographic methods in the purification of prostatic acid phosphatase (B24, O4) has already indicated that this enzyme exists in more than one molecular form, or isoenzyme. There is, in addition, immunological (S19) and starch gel electrophoretic evidence (L14, L15, S24, S31) of the existence of several forms. In order to ensure that no isoenzymes are lost during any purification, it is preferable to perform such studies on a homogenate of the whole tissue. It should be recognized that the isoenzymatic composition may not be characteristic of the prostatic cell per se, but may also represent components from blood cells, secretory ducts, connective tissue, and other sources.

Sur *et al.* (S31) subjected a concentrated aqueous extract of human prostate gland to starch gel electrophoresis in citrate buffer at pH 6.2, and obtained at least thirteen active zones. These were recovered from

the gels in four groups according to their mobilities. All four had the same pH optimum of 5.5 with disodium β-naphthyl phosphate as substrate. In this preliminary report, it was stated that the apparent Michaelis constants, stability at 47° and pH 6.2, were essentially the same for all four groups. Treatment with butanol to dissociate any possible lypoprotein–phosphatase complex, with an active protease to dissociate any possible complexes with other proteins, or with EDTA to dissociate possible metal-bound complexes failed to alter the electrophoretic pattern.

These results were confirmed to a large extent by Lundin and Allison (L14, L15), who examined the electrophoretic patterns of acid phosphatase from different organs and animal forms. We shall concern ourselves only with the results on human tissues. Since there is no statement that equal activities of acid phosphatase from different tissues were placed at the origin, it is difficult to make any definite conclusions about the patterns from the different tissues. In general, these tissues showed between 10 and 17 bands upon electrophoresis at pH 6.0 for a period of about 4 hours. Human prostate had a strong band that moved very little from the origin, and this band was not seen in the other tissues.

Smith and Whitby (S24) homogenized fresh autopsy specimens of normal human prostate, stripped of capsule, and cut into small pieces in a Waring Blendor at 4°C for 20 seconds in 4 volumes of 0.01 *M* citrate buffer (pH 6.0). The supernatant was decanted, filtered at 4°C, and stored at −20°C. When a small aliquot, 10 ml, of this homogenate was applied to a column of Sephadex G-200, and the column was eluted upward with 0.01 *M* citrate buffer (pH 6.0) containing 0.1 *M* NaCl, two peaks of acid phosphatase activity were obtained. The first peak was small and did not appear if the sample was first centrifuged at 100,000*g* for 30 minutes, and it appeared to be particle-bound enzyme. The second peak contained 90–100% of the applied activity, was always homogeneous, and appeared to consist therefore of enzyme species differing in molecular weight by less than 5%. Calibration of the column indicated a molecular weight of about 105,000.

The crude prostatic homogenate was also passed through a column of cellulose phosphate, and eluted with 0.01 *M* citrate, pH 6.0. The resulting single peak was then fractionated by DEAE-cellulose chromatography into two peaks. These two peaks (fractions II and IV) were further purified by gel filtration; they constituted 50- and 100-fold purifications from the prostatic homogenate.

There were no marked differences in the relative rates of hydrolysis of a number of phosphate esters at a concentration of 2 m*M* by the

two purified fractions. For example, the pattern of hydrolysis on some of these esters by fraction I was: *p*-nitrophenyl phosphate, 100; naphthyl 1-phosphate, 145; naphthyl 2-phosphate, 135; glucose 1-phosphate, 2; β-glycerophosphate, 60. The corresponding pattern of hydrolysis of these esters by fraction II was 100, 135, 126, 5, 83. L-(+)-tartrate at a concentration of 5 m*M* inhibited the hydrolysis of all esters equally by fraction I and fraction IV. The extent of inhibition was 90–100% for all the phosphate esters, except for 70–80% for the naphthyl phosphate esters. The optimal pH was 4.5 for *p*-nitrophenyl phosphate and about 6.0 for β-glycerophosphate and adenosine 5′-monophosphate, regardless of whether fraction II or fraction I was used.

Starch gel electrophoresis of prostatic homogenate was carried out at 4° in citrate buffer, pH 5.0, 0.5 *M* and 0.1 *M* in the cathode and anode vessels, respectively, and 5 m*M* in the gel. An overall potential of 200 V, giving a current of 35 mA was applied for 20 hours. About 20 bands were usually obtained. All bands were almost totally inhibited by 5 m*M* L-(+)-tartrate. When a sample of homogenate was digested with neuraminadase for varying periods of time, there was a progressive disappearance of the fastest bands (11–20), until the bulk of the enzyme activity was compressed into bands 3–10, after which these bands were much more slowly digested, and bands 1 and 2 increased in prominence. The results indicated that the enzyme could undergo progressive removal of acidic (probably neuraminic acid) groups. It would seem, therefore, that the electrophoretic heterogeneity of the enzyme arises from a single enzyme protein bearing a variable number of acidic residues.

The role of neuraminic acid in the heterogeneity of acid phosphatase from the human prostate gland has been studied more recently by Ostrowski and his associates (O5). Slices of frozen prostate were immersed in 3 volumes of 0.01% aqueous Tween 80 solution and left in the cold room overnight with slow mixing, filtered, and squeezed through gauze. The liquid was centrifuged at 100,000*g* for 60 minutes; the supernatant was dialyzed for 48 hours against distilled water, and then concentrated. This concentrate gave a single peak of acid phosphatase activity during ultracentrifugation in sucrose gradient, during filtration on Sephadex G-100; or on agar-gel suspensions. The enzyme activity was assayed by its action on 0.02 *M* β-nitrophenyl phosphate. These results indicated that acid phosphatase was a relatively homogeneous protein or else composed of molecules with molecular weights not differing from each other by more than 5%. Earlier studies with gel filtration (O3, S24) had indicated an average molecular weight of about 100,000.

As has been noted in the section on the purification of prostatic phosphatase, earlier investigators had observed that chromatography with Sephadex G-200 or with DEAE-cellulose yielded two peaks of enzyme activity (S24). Ostrowski *et al.* (O5) confirmed these findings. A 5-ml sample of a prostatic extract was applied to an equilibrated DEAE-cellulose and eluted with phosphate buffer of increasing concentrations and decreasing pH. Fraction I, representing 70–80% of the total activity eluted, came off with 0.05 *M* phosphate buffer at pH 6.5. At about pH 6.0 and a somewhat higher concentration of buffer, fraction II, containing the enzyme activity remaining in the column, was eluted. These two fractions were collected, concentrated, and filtered separately on a column with Sephadex G-100. These two fractions were mixed and rechromatographed on a column of DE_{52}-cellulose. Elution with phosphate buffer of increasing concentration and decreasing pH again resulted in two distinct fractions.

These two fractions, designated as enzyme I and enzyme II, were subjected to isoelectric focusing, and each gave at least four active peaks. Enzyme I yielded fractions with isoelectric peaks ranging from pH 4.8 to 5.2, and enzyme II gave four fractions with peaks ranging from 4.05 to 4.60.

When these two acid phosphatases were mixed and digested with neuraminidase and were then submitted to isoelectric focusing, one single symmetrical peak of activity was obtained at a pH of about 6.15. It was apparent that treatment with neuraminidase abolished the electrophoretic heterogeneity of these two enzymes. The splitting off of neuraminic acid (NANA) produced no appreciable change in the enzymatic activity of either acid phosphatase I or II. The isolated enzymes were hydrolyzed by trichloroacetic acid and showed liberations of 31 ± 2.8 and 40 ± 8 moles of NANA per 100,000 g of enzyme protein. In the case of enzyme I, the liberation by acid was about 25% higher than that by neuraminidase. These results indicate rather clearly that the large number of isoenzymes of prostatic acid phosphatase which have been demonstrated by gel electrophoresis or isoelectric focusing differ from each other in the number of neuraminic acid residues attached to essentially the same protein molecule.

3.2.4. *Kinetics of Human Prostatic Acid Phosphatase*

Using an approximately 300-fold purified preparation of prostatic acid phosphatases, obtained essentially according to the procedure of London and Hudson (L10), Tsuboi and Hudson (T3) undertook several types of kinetic studies. These investigators observed that the purified preparation of the prostatic acid phosphatase was highly unstable in

dilute solution and was inactivated rapidly, that a relationship existed between the inactivation process and time of shaking the enzyme preparation and the temperature, and that a surface-active agent like Triton X-100 or various proteins prevented the inactivation.

A second important factor governing the reaction velocity was the presence of various ions. The usual Lineweaver-Burk plot of reciprocal of reaction velocity against the reciprocal of substrate (sodium β-glycerophosphate) yielded a straight line relationship only with dilute substrate concentrations. With increasing substrate concentration, the reaction velocity and consequently the slope increased by an increment in excess of that predicted by theory. Tsuboi and Hudson (T3) explained these effects by assuming that the change in the concentration of substrate, including the cation Na^+, constituted changes in the ionic environment and hence accounted for the deviations at the higher levels. With decreasing substrate concentrations, that is, to levels of 0.005 *M* and below, the ionic differences became negligible in the presence of relatively high buffer concentrations, as, for example, 0.15 *M* acetate at pH 5.5. Tsuboi and Hudson (T3) also found that citrate buffer, 0.05 *M*, or acetate buffer, 0.15 *M* with 0.01 *M* EDTA gave substantially higher velocities than acetate alone, and they attributed this effect to the abolition of inhibitory action by contaminant traces of heavy metals.

When these factors were taken into account, and velocities were determined at dilute concentrations of substrate and in the presence of 0.1 *M* acetate, pH 5.5, as buffer and 0.01 *M* EDTA, it was possible to determine the Michaelis constants for different substrates. At 37°C these values were as follows: α-glycerophosphate, 3.1 m*M*; β-glycerophosphate, 2.4 m*M*; yeast adenylate, 0.25 m*M*; phenyl phosphate, 0.15 m*M*. The corresponding values for V_{max}, the velocity at infinite substrate concentration, were expressed as micrograms of phosphate liberated per minute: 0.9, 1.0, 1.0, and 1.0. With phenyl phosphate as substrate, L-(+)-tartrate was found to be a strong competitive inhibitor, with K_i equal to 0.63×10^{-4} *M*. The enzyme was also reversibly inactivated by cupric and ferric ions and by the thiol reagent, *p*-chlormercuribenzoate.

Some years later, Nigam *et al.* (N3) undertook a kinetic study with preparations that represented an approximately 20- to 30-fold purification (D3). They first presented the time courses of hydrolysis of phenyl phosphate, nitrophenyl phosphate, and β-glycerophosphate, the first two at an initial concentration of 0.0043 *M* and the last, β-glycerophosphate, at a concentration of 0.0028 *M*. Although the hydrolyses of phenyl phosphate and nitrophenyl phosphate were of zero order for approximately the first 25% of the reaction, that of β-glycerophosphate was of this order for only the first 10%, the reaction velocity decreasing

progressively after that. The plot of reaction velocity against enzyme concentration exhibited straight lines for phenyl phosphate and nitrophenyl phosphate and a slightly curved one for β-glycerophosphate. Direct proportionality of a correctly chosen measure of reaction velocity enzyme concentration is a fairly universal characteristic (B20).

The pH activity curves were of interest. The optimum pH for hydrolysis of phenyl phosphate was 4.9, 5.0, and 5.0 in acetate, citrate, and Tris·HCl buffer solutions, respectively. For nitrophenyl phosphate, the corresponding values were 4.9, 4.7, and 5.5, and for β-glycerophosphate, the values were, respectively, 5.5, 5.7, and a range of 5.0 to 6.0. The Michaelis constants, determined at these optima and in acetate buffer, were 0.75 m*M* for phenyl phosphate, 0.81 m*M* for nitrophenyl phosphate, and 4.0 m*M* for β-glycerophosphate. In the presence of citrate buffer, the corresponding values were 0.091 m*M*, 0.3 m*M*, and 2.0 m*M*. It may be seen that the values for β-glycerophosphate were in fairly good agreement with those obtained by Tsuboi and Hudson (T3), whereas the discrepancies for phenyl phosphate were somewhat greater. With citrate as buffer, the Michaelis constants were 0.091 m*M* for phenyl phosphate, 0.31 m*M* for nitrophenyl phosphate, and 2.0 m*M* for β-glycerophosphate.

Of various monocarboxylic and dicarboxylic acids tested at a concentration of 0.01 *M*, only oxalate, saccharate, and L-(+)-tartrate showed substantial inhibition of the action on all three substrates. For example, oxalate inhibited these actions in the standard assay method as follows: phenyl phosphate, 26%; *p*-nitrophenyl phosphate, 41%; β-glycerophosphate, 72%. The comparable inhibitions by saccharate were 42, 64, and 91% and those by L-(+)-tartrate were 96, 97, and 100%. Whereas other carboxylic acids like maleate, glutamate, malonate, and glucuronate did not inhibit the hydrolysis of phenyl phosphate *p*-nitrophenyl phosphate, the inhibition of the hydrolysis of β-glycerophosphate was fairly substantial, ranging from about 40–60%. The inhibition by L-(+)-tartrate was studied throughout a range of substrate concentrations and yielded the following values for the inhibition constant, K_i: 0.95×10^{-5} *M* with phenyl phosphate as substrate; 4.5×10^{-5} *M* with nitrophenyl phosphate and 2.4×10^{-5} *M* with β-glycerophosphate. The value with nitrophenyl phosphate was essentially that, 3.4×10^{-5} *M*, reported by Kilsheimer and Axelrod (K3).

There is no information concerning the isoenzymatic composition of the purified prostatic phosphatases that were used in the preceding kinetic studies. Nor do there appear to be any kinetic studies on the individual isoenzymes. The possibility exists that substantial differences in kinetic characteristics, such as the value for K_i, for L-(+)-tartrate,

reported by different investigators may reflect differences in the isoenzymatic composition of the purified prostatic acid phosphatases which they employed.

3.3. Human Erythrocytic Acid Phosphatase

3.3.1. *Introduction*

The presence of acid phosphatase in the human erythrocyte was recognized in 1934 (D4) and properties of this enzyme were studied for almost thirty years (A4, K6, T1, T2, T4, T5) before its role in human genetics was revealed (H13). This role will be described in detail later. The properties of crude preparations of erythrocytic acid phosphatase have been previously noted in this review. At this point, we shall describe methods of purification, and the nature of the isoenzymes, particularly as they are related to the phenomenon of polymorphism.

3.3.2. *Purification of Human Erythrocytic Acid Phosphatase*

Tsuboi and Hudson (T2) described a 1500-fold purification. One liter of red cells from outdated blood was thoroughly washed free of leukocytes and plasma proteins with 1% saline, hemolyzed with four volumes of distilled water, and stirred thoroughly with 400 ml of calcium phosphate gel suspension, containing approximately 45 mM tricalcium phosphate per liter. The gel was removed by centrifugation, washed twice with small volumes of distilled water and then discarded. The combined supernatant and washes which contained almost all the original enzyme activity, was now mixed with 1600 ml of calcium phosphate gel suspension. The gel, which had absorbed 90% of the enzyme, was washed by repeated centrifugation until the washings were colorless. The washed gel was resuspended evenly with 1 liter water and mixed for several minutes with 1 liter of 0.3 M acetate–0.03 M citrate buffer, pH 4.5. The mixture was then centrifuged, and the supernatant eluate contained 60–70% of the adsorbed enzyme. At this stage, the degree of purification, as compared with crude hemolysate, was between 150- and 200-fold on the basis of nitrogen determination.

The enzyme solution was then treated with ammonium sulfate to 55% saturation. The precipitated enzyme was centrifuged, dissolved in a minimal volume of water (about one seventy-fifth that of the eluate) and dialyzed overnight against several hundred volumes of 0.01 M acetate, pH 5.0. Any precipitated protein was centrifuged off; the color of the resulting solution was a dark brown red due chiefly to the presence of catalase and hemoglobin. The degree of purification was approximately 400-fold that of the crude hemolysate.

The dialyzed enzyme solution was now subjected to a repetition of the preceding procedures: admixture of sufficient calcium phosphate gel to adsorb protein but leave the enzyme in solution; centrifugation and addition of more gel to the supernatant to adsorb the enzyme; elution of the enzyme from the gel with a mixture of 0.15 *M* acetate and 0.015 *M* citrate at pH 4.5; addition of solid ammonium sulfate to the eluate to 55% saturation and precipitation of the enzyme. At this stage, the purifications ranged from 650- to 1100-fold with a recovery of approximately 20–30% of the activity present in the crude red cell hemolysate. Solution of this precipitate, dialysis; treatment with solid ammonium sulfate; and collection of the precipitate appearing between 40 and 55% saturation yielded a preparation that represented a 1500-fold purification. The preparations were stable when left sedimented in the ammonium sulfate sclution.

A much purer preparation of acid phosphatase from horse erythrocytes was obtained by Ito *et al.* (I2) by adding the DEAE-chromatography procedure to the method of Tsuboi and Hudson (T2). Since this procedure may be applicable to human erythrocytes, it will be mentioned briefly. One liter of horse erythrocytes was washed and lysed by the addition of 4 liters of distilled water. One liter of calcium phosphate gel suspension was added to the hemolysate to remove most of the nonenzymatic protein, and the mixture was centrifuged. Five liters of the gel suspension were added to the supernatant, resulting in the adsorption of the enzyme. The enzyme was eluted with citrate–acetate buffer, pH 4.5, and solid ammonium sulfate was added to the eluate up to 60% saturation. The precipitate was collected, dissolved in 40 ml of water, dialyzed against water at 5°C for 10 hours, and again subjected to calcium phosphate gel adsorption, elution, and precipitation with solid ammonium sulfate to 60% saturation.

The precipitate was dissolved in a minimal volume of water (4 ml) and dialyzed against water; the resulting solution was applied to a DEAE-cellulose column which had been equilibrated with 0.01 *M* sodium pyrophosphate buffer containing 0.08% of the detergent Emargin 810. A linear gradient elution was then carried out with 0.01 to 0.2 *M* sodium phosphate buffer, pH 6.0. The eluate containing the enzyme was freed of buffer by passage through a Sephadex G-50 column, previously washed with 0.001 *M* mercaptoethanol and 0.08% Emargin 810. The specific activity of the material prior to application on the DEAE column was 15.0, as compared with the specific activity of 0.0139 in the crude hemolysate. This represented a 1080-fold purification, of the same order as that reported by Tsuboi and Hudson (T2). The first part of the enzyme peak coming through the DEAE-cellulose column con-

tained 8.4% of the enzyme originally present in the crude red cell hemolysate and had a specific activity of 120. This represented an approximately 9000-fold purification.

3.3.3. *Isoenzymes of Human Erythrocytic Acid Phosphatase*

In the course of studying chromatography of various proteins on the anion-exchange resin Dowex 2, Boman and Westlund (B25) observed that human erythrocytic acid phosphatase was eluted in two peaks. Several years later, in 1962, Angeletti and Gayle (A11) studied the chromatography of a centrifuged and dialyzed 1:5 human red cell hemolysate on a DEAE-cellulose column. Twenty to 25 ml of the hemolysate was applied to the column; washing with phosphate buffer removed practically all of the hemoglobin. A parabolic gradient salt elution, increasing to a final concentration of 1 *M* NaCl, was started after an effluent volume of 200 ml had been collected. At approximately an effluent volume of 400 ml and an NaCl concentration of about 0.1 *M*, the acid phosphatase began to emerge. This enzyme consistently appeared in three fairly well separated peaks. Angeletti and Gayle (A11) were able to elicit some differences in the characteristics of these peaks. With *p*-nitrophenyl phosphate as substrate and citrate as buffer, peak 1 showed optimal activity at pH of about 4.4, whereas peaks 2 and 3 had a pH optimum of about 5.5. Again, the acid phosphatase in peak 1 could be inhibited by 0.02 *M* tartrate to the extent of 40%, whereas peaks 2 and 3 were not inhibited, even up to concentrations of 0.04 *M* tartrate. Prostatic phosphatase, it will be recalled, is almost completely inhibited by 0.01 *M* L-(+)-tartrate.

Georgatsos (G1) failed to obtain any fractions upon applying whole hemolysates to Sephadex G-75 or G-100. However, when he precipitated the acid phosphatase with acetone, washed the precipitate twice in acetone, then extracted the resulting dry powder with 0.14 *M* NaCl, he obtained an active preparation of acid phosphatase. Application of aliquots of this extract to Sephadex G-75 and elution with 0.14 *M* NaCl resulted in two peaks. The first peak had two pH optima, one at pH 5.0 and another at pH 6.0. It was activated by Mg^{2+} optimally at a concentration of 6.6 m*M*. The second peak had a pH optimum at 5.0 and was not affected by Mg^{2+}. Conversely, fluoride at a concentration of 10 m*M* inhibited the enzyme activity in the first peak to the extent of 47% but did not affect that in the second. As Georgatsos (G1) has pointed out, the conflicting results obtained by different investigators may be due to the change in proportion of these two components as purification proceeds from the crude hemolysate.

In 1963, Hopkinson *et al.* (H13) observed that, when human red cell

hemolysates were subjected to starch gel electrophoresis, more than one zone of acid phosphatase activity were present. At this time, five patterns, A, BA, B, CA, and CB were detected and were described qualitatively in terms of the relative activity and migration of the zone toward the anode. For example, in type A, the "fast" zone was generally clearly defined and well separated from the "intermediate" zone. In type BA, the "fast" zone was less sharply defined and appeared to merge with the "intermediate" zone which was very much more intense than in type A. There was also a trace of a "slow" zone. Type B showed no "fast" zone, but had a very strong "intermediate" zone and a fairly intense "slow" zone. These patterns indicated several acid phosphatase variants and implied the existence of a new type of human polymorphism. This subject will be considered in greater detail in Section 5.

3.3.4. *Kinetics and Other Properties of Human Erythrocytic Acid Phosphatase*

In 1953, Tsuboi and Hudson (T1) considered some kinetic characteristics of crude red cell acid phosphatase preparation, similar to that used by Abul-Fadl and King some years earlier (A3, A4). Unlike the latter investigators, Tsuboi and Hudson (T1) observed only one pH optimum, around 5.5, with phenyl phosphate, α- and β-glycerophosphates or yeast adenylic (probably a mixture of adenosine 3′- and 2′-monophosphates) as substrates. The enzyme was activated by added magnesium with optimal effect being obtained at a concentration of 0.01 M. Neither prolonged dialysis nor precipitation by acetone resulted in preparations that showed a greater activation by magnesium. The relative rates of hydrolysis of various substrates by nondialyzed hemolysate at pH 5.5 and 0.01 M magnesium and expressed as milligrams of phosphorus liberated per hour per milliliter of red blood cells may be illustrated by the following results: phenyl phosphate, 4.57; α-glycerophosphate, 2.74; β-glycerophosphate, 0.51; yeast adenylic acid, 0.21. Dialyzed hemolysates did not show any significant difference.

In 1948, Axelrod (A17) and in 1950, Meyerhof and Green (M7) showed that acid phosphatases from various sources were capable of mediating direct transfers of phosphate from suitable donors to suitable acceptors. Tsuboi and Hudson (T1) investigated this phenomenon by determining the amounts of phenol and phosphorus liberated from phenyl phosphate in the presence of increasing concentrations of an acceptor such as glycerol or methanol. For example, at 0.0069 M phenyl phosphate and 0.69 M glycerol, 153 μmoles of phenol and 71 μmoles of phosphate were found to be liberated per hour per milliliter of red blood cells. These results indicated a transfer of 82 μmoles of phosphate to

TABLE 1
COMPARATIVE ACTIONS OF HIGHLY PURIFIED PREPARATIONS OF HUMAN ERYTHROCYTIC AND PROSTATIC ACID PHOSPHATASES ON VARIOUS SUBSTRATES[a]

Substrate	Relative activities as percent of maximum	
	Erythrocytic acid phosphatase	Prostatic acid phosphatase
Phenyl phosphate	100	100
α-Glycerophosphate	33	69
β-Glycerophosphate	2	84
3-Phosphoglycerate	4	0
Glucose 1-phosphate	1	19
Glucose 6-phosphate	0	0
Ribose 5-phosphate	8	11
Adenosine 5'-phosphate	3	60
Adenosine triphosphate	0	0
Sodium pyrophosphate	1	0
Diphenyl phosphate	0	0

[a] Based on data of Tsuboi and Hudson (T3, T5).

glycerol. The extent of phosphate transfer increased with the concentration of glycerol.

A 1000-fold purified preparation of erythrocytic acid phosphatase was used later by Tsuboi and Hudson (T5) in a reinvestigation of the kinetic properties. The pH activity curve now had a broader maximum centering around pH 6.0. Mg^{2+} no longer had any activating effect at any pH level. The rate of action varied greatly with the substrate. Table 1 shows the comparative actions of the highly purified preparations of erythrocytic acid phosphatase and a similarly highly purified preparation of prostatic acid phosphatase. The concentration of each substrate was 0.01 *M*, and all velocities were determined in the absence of magnesium, except for ATP and $Na_4P_2O_7$ which were tested with 0.01 *M* Mg^{2+}.

Reaction velocities were determined at various concentrations of α-glycerophosphate and of phenyl phosphate at pH 5.5 and 37° with 0.1 *M* acetate as buffer and 0.001 *M* EDTA. A Lineweaver-Burk plot yielded a value of 7 m*M* for the Michaelis constant with α-glycerophosphate as substrate and 0.9 m*M* with phenyl phosphate as buffer. It will be recalled that the corresponding values for human prostatic phosphatase were 3.1 m*M* and 0.15 m*M* according to Tsuboi and Hudson (T3). Nigam *et al.* (N3) had obtained a value of 0.75 m*M* for phenyl phosphate. In view of the experimental errors inherently involved in the determination of Michaelis constants leading frequently to coefficients

of variation of 25–30% (N4), it would appear that no marked distinction can be made between the values for the Michaelis constants for human erythrocytic acid phosphatase and those for the prostatic enzyme.

In contrast to the marked inhibition of prostatic phosphatase by fluoride (A4) and L-(+)-tartrate (A4, F1, K3), neither the acid phosphatase present in crude hemolysates or in a highly purified preparation from such hemolysates is inhibited by these compounds (A4, T5). Tsuboi and Hudson (T5) also studied the effect of temperature on the hydrolysis of α-glycerophosphate by the erythrocytic enzyme at the optimum pH, 6.0, for this substrate. Using the Arrhenius plot (A15, B21), namely, log velocity against the reciprocal of the temperature, Tsuboi and Hudson (T5) obtained two straight lines that appeared to intersect at 26°C and which yielded energies of activation of 13,000 calories at the lower temperatures up to about 26°C, and 9600 calories for the range from this temperature up to about 50°C. The corresponding values for the energy of activation for prostatic acid phosphatase were 12,000 calories up to 26°C, and 8000 calories above this temperature. The Arrhenius equation has been found to hold, that is, E is constant as T is varied, for a great many chemical reactions (B20), and its reported failure to hold in enzymatic reactions has been shown in many cases to be due to the incorrect use of proper measures of reaction velocity (B20). When correct measures were used, a straight line is obtained between the log reaction velocity and the reciprocal of the absolute temperature in accordance with the Arrhenius equation (B20, B21). It is, therefore, possible that the results reported by Tsuboi and Hudson (T5) reflect the use of improper measures of reaction velocity.

An alternate explanation is also possible. Using proper measures of reaction velocity in studying the effect of temperature on the fumarase activity, Massey (M6) obtained a straight line relationship with sodium L-malate as substrate, that is, for the dehydration reaction. In contrast, with sodium fumarate as substrate, that is, for the hydration reaction, a straight line relationship held only at pH 7. At higher or lower pH levels, the relationship could best be described at any given pH by two straight lines intersecting at a critical temperature yielding different energies of activation. At pH levels higher than 7.0, the activation energy at lower temperatures was lower than that at higher temperature, whereas at pH levels lower than 7.0, the activation energy at the lower temperatures was greater than those at the higher temperatures. It is, therefore, also possible that the temperature effects described by Tsuboi and Hudson (T5) resemble those by Massey (M6) and may be explained by the formulation of Kistiakowsky and Lumry (K7) that deviations from the Arrhenius relationship are the result of low tem-

perature reversible inhibition by one or more constituents of the reaction mixture.

Like many other purified enzymes, a 1000- to 1500-fold purified preparation of erythrocytic acid phosphatase is inactivated, particularly when present in dilute solutions. Investigations by Tsuboi and Hudson (T4) showed that two separate phenomena were responsible for the instability of the purified preparation. First, the addition of very small quantities of synthetic nonionic detergents, like Triton X-100, resulted in a complete stabilization of the enzyme against inactivation due to surface forces. Second, the enzyme was found to be rapidly inactivated by trace amounts of heavy metals which were present or introduced as a contaminant through the use of dialyzing membranes or various reagents. This susceptibility to inactivation suggested the presence of sulfhydryl groups in the enzyme.

3.4. Human Leukocytic Acid Phosphatase

The acid phosphatase activity of leukocytes was studied by Valentine and Beck (B8, V1) in 1951. There appear, however, to have been no significant attempts to purify the enzyme from this source, or to describe its characteristics. Recently, Szajd and Pajdak (S32) indicated the isoenzyme characteristics of leukocyte acid phosphatase, and Li and his associates (L7, L8) studied this problem in greater detail. They suspended a leukocyte preparation, carefully separated from blood, in 5% Triton X-100 to yield a final concentration of 10×10^6 cells per milliliter and subjected the suspension to six cycles of alternate freeze–thaw treatment. The suspension was then centrifuged at 1000*g* for 15 minutes at 4°C, and the supernatant was used for electrophoretic studies. Specimens centrifuged at 100,000*g* for 15 minutes gave the same results. Electrophoresis was carried out at 4°C for 60 minutes on a 7.5% acrylamide gel matrix containing 0.5% Triton X-100 at pH 4.0 with a current of 4 mA per tube. The substrate was α-naphthyl phosphate.

The values for the normal leukocyte acid phosphatase activity and the normal isoenzyme pattern will be described in connection with the alteration of these in various hematologic and hematopoietic disorders.

3.5. Liver Acid Phosphatase

3.5.1. *Introduction*

Liver acid phosphatase has been of particular interest since the demonstration by de Duve (D7, D8, D9, D10) that acid phosphatase and other hydrolytic enzymes were enclosed in an intracellular structure, the lysosome, of the liver and played an important role in the intra-

cellular digestion of foreign and endogenous material. This aspect of acid phosphatase will be considered in detail later in this review.

The purification of acid phosphatase from the human liver and the description of its properties do not appear to have been accomplished. Partly, this may be due to the inherent difficulty of obtaining normal, fresh human material in amounts substantial enough for purification. However, because of the cellular and physiological importance of acid phosphatase, it is advisable to describe in the present section the purifications of the enzyme from rat and bovine liver. Moreover, since these purifications were accomplished with the awareness that acid phosphatase from this source might be present in multiple molecular forms, the descriptions will naturally involve a consideration of the isoenzymes and their properties.

3.5.2. *Rat Liver Acid Phosphatase*

The electrophoretic characteristics of rat liver acid phosphatase were considered by Barka in 1961 (B3). He prepared 10% distilled water homogenates from livers of rat, perfused *in situ* with cold Ringer's solution. After freezing at −68°C and thawing five times, the homogenates were centrifuged at 105,500*g* for 60 minutes and the supernatants, excluding the top lipid-rich layer, were used for electrophoresis on polyacrylamide gels. The soluble acid phosphatases in the supernatant represented 60% of the activity of the total homogenate. Gomori's acid phosphatase technique with β-glycerophosphate as substrate (G7), the post-incubation coupling azo dye method with sodium 6-benzoyl-2-naphthyl phosphate as substrate (R10), and several other methods were employed in eliciting three bands of acid phosphatase activity. Employing the supernatant obtained by centrifuging a 1:3 rat liver homogenate at 100,000*g* for 60 minutes, Moore and Angeletti (M9) were able to separate by DEAE-cellulose chromatography three major and one minor peaks of acid phosphatase activity. Arsenis and Touster (A16) reported that a partially purified rat liver lysosomal acid phosphatase could be resolved into two enzyme components, a 5′-nucleotidase and a sugar phosphate phosphohydrolase.

A 336-fold purification by column chromatography was achieved by Brightwell and Tappel (B32) from lysosomes obtained by differential and density sucrose gradients. The lysosomes were frozen and thawed several times, then centrifuged. The resulting soluble acid phosphatase fraction was dialyzed against suitable buffers and then applied to a DEAE-cellulose column or to a CM-cellulose column. Each column was eluted with a linear 0–1 *M* NaCl solution, the former at pH 7.2 and the latter at pH 5.6. Two peaks of acid phosphatase activity were ob-

tained in each case. The first peak from the DEAE-cellulose column showed a 336-fold purification. The acid phosphatase from the CM-cellulose eluate was inhibited by 1.4 m*M* L-(+)-tartrate to the extent of 80% with *p*-nitrophenyl phosphate as substrate and to 97% when β-glycerophosphate was the substrate.

Purification and crystallization of acid phosphatase from rat liver has been reported by Igarashi and Hollander (I1). All procedures were carried out at 4°C, and all solutions contained 5 m*M* 2-mercaptoethanol and 1 m*M* EDTA. Livers from freshly killed rats were immediately homogenized with 50% glycerol in a Waring Blendor for 3 minutes, and the homogenate was centrifuged at 8000 rpm for 20 minutes. About 90% of the activity was recovered in the supernatant; this was adjusted to pH 5.0 with 1 *M* acetic acid and stirred for 30 minutes. The precipitate that formed was separated by centrifugation and discarded. The supernatant fluid was dialyzed overnight against 5 m*M* acetate buffer, pH 5.0, and the resulting precipitate was also removed by centrifugation and discarded. The dialyzed material was treated with ammonium sulfate so as to obtain the fraction precipitating between 0.5 and 0.8 saturation.

This precipitate, representing a 6.9-fold purification and 53% recovery, was dissolved in a minimum quantity of 0.01 *M* sodium acetate buffer, pH 5.0, and applied to a Sephadex G-75 column equilibrated with 0.01 *M* sodium acetate buffer. Elution with the same buffer gave a single peak of enzyme activity, but only the aliquots containing higher enzyme activity were pooled and precipitated by addition of ammonium sulfate between 0.5 and 0.8 saturation. The resulting precipitate was dissolved in 5 m*M* imidazole buffer, pH 7.1, and dialyzed against the same buffer. The enzyme solution was then chromatographed on a DEAE-cellulose column and eluted by a linear gradient composed of a mixture of the buffer and 0.5 *M* NaCl. Two peaks of enzyme activity were obtained, pooled separately and concentrated by precipitation with 0.8 saturated ammonium sulfate.

The first peak of enzyme solution was dialyzed against 10 m*M* sodium succinate buffer, pH 6.0, without EDTA and with 2-mercaptoethanol, then applied to a hydroxyapatite column equilibrated with the same buffer. Stepwise elution with ammonium sulfate in this buffer yielded a single peak in 0.1 *M* ammonium sulfate fraction. The pooled enzyme was concentrated with 0.8 saturated ammonium sulfate to give 1% protein concentration in imidazole-glycylglycine buffer, pH 7.1. Addition of ammonium sulfate to between 0.5 and 0.55 saturation yielded a turbid solution which in turn yielded crystals after 24 hours at 4°C. The second peak was applied to a Sephadex G-200 column equilibrated with 0.01 *M*

sodium acetate buffer and eluted with the same buffer as a single peak, termed PII enzyme.

The properties of these two components or isoenzymes of rat liver phosphatase were similar in many respects, but different in some. Thus, the molecular weight of each was approximately 100,000. With *p*-nitrophenyl phosphate as substrate, the Michaelis constant was 0.091 ± 0.007 m*M* for the crystalline isoenzyme and 0.047 ± 0.004 for the PII component. The isoelectric points of the crystalline and PII isoenzymes were pH 7.7 and 4.5, respectively, as determined by the method of isoelectric focusing. The activity of each isoenzyme was completely inhibited by 1 m*M* L-(+)-tartrate or fluoride at a concentration of 1.0 m*M* *p*-nitrophenyl phosphate as substrate.

The finding by Igarishi and Hollander (I1) that rat liver acid phosphatase has two isoenzymes is not in agreement with the observation of Barka (B3), who found three components on polyacrylamide electrophoresis, or of Moore and Angeletti (M9), who were able to separate by DEAE-cellulose chromatography three major peaks and one minor peak of this enzyme activity. Using a lysosomal extract of rat liver Shibko and Tappel (S15) found three fractions by DEAE-cellulose chromatography.

3.5.3. *Bovine Liver Acid Phosphatase*

Heinrikson (H3) has recently submitted a purification and characterization of a low molecular weight acid phosphatase from bovine liver. Five hundred grams of fresh bovine liver, minced in a meat grinder, were extracted at 0–4°C with 1500 ml of 0.3 *M* sodium acetate, pH 5.0, containing 1 m*M* EDTA (buffer A). The suspension was stirred for 90 minutes at room temperature and then centrifuged at 4°C for 30 minutes at 15,000*g*. The supernatant, which amounted to 1400 ml, was stirred with 246 g of solid ammonium sulfate. The mixture was centrifuged for 20 minutes at 15,000*g*, and the resulting pellet was discarded. The ammonium sulfate concentration of the supernatant solution was increased to 55%. The precipitate, containing most of the enzyme, was centrifuged off, dissolved in 200 ml of 0.3 *M* acetate buffer, pH 5.0, and centrifuged to remove the sediment.

The supernatant solution (196 ml) was diluted with 4 volumes of cold 0.1 *M* sodium acetate, pH 5.0; the resulting mixture was chilled to 0° and its pH lowered to 4.15. The suspension was immediately centrifuged and the precipitate discarded. Cold 0.1 *M* Tris was added to the supernatant to bring the pH back to 5.0, and the solution was centrifuged. The supernatant was again fractionated by the addition of solid ammonium

sulfate, as described previously, so as to obtain the precipitate between 35 and 50% saturation which was centrifuged off and dissolved in 80 ml 0.1 *M* sodium acetate, 1 m*M* EDTA, pH 5.0.

The solution was divided into two 40-ml portions, and each portion was added to a column of Sephadex G-75 that had been equilibrated with 0.01 *M* sodium acetate, 1 m*M* EDTA, pH 4.8. Elution was continued in the same buffer. Gel filtration of a crude extract of bovine liver on Sephadex-75 had previously given two small peaks and a third large peak of acid phosphatase activity. Elution of the purified 35–50% ammonium sulfate fraction now gave a small peak of about 10% of the enzyme activity, no second peak, and a third peak that contained 90% of the enzyme. The third peak (acid phosphatase III) represented the low molecular weight component and constituted 30% of the total acid phosphatase present in the 15,000*g* supernatant starting material; the degree of purification was 54-fold.

The phosphatase III effluent was now chromatographed on a column of sulfoethyl Sephadex C-50. The enzyme was adsorbed, whereas a considerable portion of the nonenzymatic protein passed through; the enzyme was then washed off with a linear gradient of increasing phosphate concentrations at pH 6.0. The specific activity rose to 360-fold that of the original 15,000*g* supernatant. A small sample was subjected to acrylamide gel electrophoresis and revealed about 10–12 bands. The remainder was concentrated by ultrafiltration under N_2 to about 30 ml, and solid ammonium sulfate was added to 75% saturation. The precipitate was separated by centrifugation, dissolved in 1 ml of 0.01 *M* sodium acetate, pH 5.0, containing 0.1 *M* NaCl and 1 m*M* EDTA. Ammonium sulfate was removed by gel filtration on a column of Sephadex G-25. The enzyme solution was added to a column of sulfoethyl Sephadex C-50 and eluted with a linear gradient of increasing NaCl concentration and an increasing, nonlinear gradient of pH. The phosphatase emerged well behind the bulk of the eluted protein; the protein and activity curves of the enzyme peak were coincident. The specific activity was 4300-fold that of the original 15,000*g* supernatant. Acrylamide electrophoresis revealed a single band.

The liver acid phosphatase III thus isolated had a molecular weight of 14,000 daltons as determined by filtration through a column of Sephadex G-75 that had been calibrated with markers of known molecular weight, and a molecular weight of 16,500 daltons on the basis of sedimentation equilibrium analysis. With *p*-nitrophenyl phosphate as substrate, the pH optimum was 5.5 and the Michaelis constant was 0.75 m*M*. The stability of the enzyme at 25° was dependent on pH and

the nature of the buffer. The presence of Mg^{2+} or mercaptoethanol in the incubation mixtures led to rapid inactivation of the enzyme, whereas EDTA exhibited a stabilizing effect.

The substrate specificity of liver acid phosphatase III was of particular interest. With the activity with *p*-nitrophenyl phosphate set at 100 and under standardized conditions, the relative activities with the following substrates were: flavin mononucleotide, 68; galactose 6-phosphate, 39; glucose 1-phosphate, 14. The relative activities with other hexose or ribose phosphates were 1 to 2, and those with α-glycerophosphate and β-glycerophosphate were 3 and 0, respectively. It will be recalled that for prostatic phosphatase, the ratio of the activity with β-glycerophosphate as substrate to that with *p*-nitrophenyl phosphate was considerably higher—about 60%. These relative activities of different tissue phosphatases are of importance in understanding the sources of serum acid phosphatase activities in various diseases.

3.6. Spleen Acid Phosphatase

In 1957, Singer and Fruton (S22) obtained from beef spleen a preparation that represented a 50-fold purification of phosphoprotein phosphatase, phosphoamidase, and phenylphosphatase, as measured by the hydrolysis of the corresponding appropriate substrates at pH 6.0. This type of preparation was purified further by Glomset (G5) by dissolving 1 g of the enzyme preparation in 4 ml of distilled water, filtering through a column containing 20 g of TEAE-cellulose in equilibrium with 0.01 *M* Tris·HCl, and displacing it with the same buffer solution. The enzyme, phosphoprotein phosphatase, emerged in the first protein peak; the succeeding peaks contained little activity. This step represented a 4.8-fold purification. The enzyme material was then subjected to three consecutive electrophoresis at pH 5.6 on "Pevikon," a polyvinyl acetate–polyvinyl chloride supporting medium. The third electrophoresis showed a single protein peak which contained the enzyme, and represented a 17-fold purification of the Singer and Fruton (S22) preparation. Although this procedure was designed to purify phosphoprotein phosphatase activity, the final preparation also showed phosphoamidase and phosphatase activity at pH 6.0 as determined on *p*-nitrophenyl phosphate as substrate. Its action on glycerophosphate or bis(*p*-nitrophenyl) phosphate was negligible.

In 1966, Chersi *et al.* (C1) submitted a procedure for isolating a highly purified preparation of acid phosphatase from hog spleen. The starting material was a crude spleen nuclease II which contained 110–120 units of acid phosphatase per kilogram of ground spleen and had a specific activity of 0.2–0.3. Chromatography on DEAE-Sephadex A-50 yielded

two major protein peaks of which the first contained most of the acid phosphatase. The specific activity had now risen to 1.65. This fraction was chromatographed on hydroxyapatite; the eluate consisted of four major protein peaks of which the second contained phosphodiesterase and the third, acid phosphatase. The specific activity had risen to 7.05.

The third step consisted in loading the acid phosphatase peak on a Sephadex G-100 column equilibrated and then eluted with 0.1 *M* acetate buffer, pH 5.6. The acid phosphatase came off before the main protein peak and had a specific activity of 78.4. For the fourth and final step, the active fraction was applied to a CM-Sephadex C-50 column equilibrated with 0.1 *M* acetate buffer, pH 5.6, and was eluted by a gradient, 0.1 to 0.3 *M*, of acetate buffer at a molarity of about 0.26 *M*. It was again applied to the same column and eluted at 0.26 *M* acetate buffer.

This final acid phosphatase preparation had a specific activity of 468 and represented an approximately 1900-fold purification of the acid phosphatase in the starting crude spleen nuclease II. It contained no acid deoxyribonuclease, acid ribonuclease, exonuclease, and phosphodiesterase activities that could be detected in a 0.1-ml sample after 2 hours of incubation with the appropriate substrate. The relative rates of hydrolysis of various substrates were as follows: *p*-nitrophenyl phosphate, 100; 5′-AMP, 63; β-glycerophosphate, 60; ATP, 0. With *p*-nitrophenyl phosphate as substrate, the pH optimum was broad and lay between pH 3.0 and pH 4.8. The Michaelis constant at 37°C was 7.25×10^{-2} m*M*. Phosphate and chloride ions acted as competitive inhibitors.

3.7. Human Placental Acid Phosphatase

In 1959 Ahmed and King (A5) determined the properties and activities of placental acid phosphatase. The tissue was washed free of blood by perfusion with 0.9% NaCl, blotted, minced, and homogenized with an equal volume of water in a Waring Blendor for 2 minutes and centrifuged to obtain the supernatant solution. (The time and speed of centrifugation were not given.) The activity was expressed as milligrams of phenol liberated in 1 hour from phenyl phosphate at pH 4.9. The mean activity in a series of 10 placental extracts was 2.4 units per gram of wet tissue. Since the addition of formaldehyde in the assay system inhibited the activity to the extent of from 0 to 100% in the individual placental extracts and, on the average, of about 50%, it was concluded that the placental acid phosphatase consisted of two components.

More recently, DiPietro and Zengerle (D13) studied the properties of acid phosphatase obtained from homogenates of perfused placentas centrifuged at 600*g* for 5 minutes to eliminate cellular debris. The resultant supernatant was then centrifuged at 96,600*g* for 45 minutes in

the Spinco No. 50 rotor. This high speed supernatant, which contained about 40% of the total acid phosphatase, was chromatographed on Sephadex G-200. Three peaks of acid phosphatase activity, designated as phosphatases I, II, and III, were obtained. The molecular weights were estimated by sucrose density gradient centrifugation and were, respectively: >200,000; 105,000, and 35,000.

When the homogenate was centrifuged at 20,000*g* (time not given) and the pellet washed with 0.25 *M* sucrose, resedimented and rehomogenized in 0.25 *M* sucrose containing 1% (w/v) of Triton X-100, the supernatant resulting from centrifugation at 100,000*g* contained about half of the acid phosphatase bound to the particles. As will be seen presently, this phosphatase, designated as isoenzyme P for convenience, resembled acid phosphatase II in several respects.

DiPietro and Zengerle (D13) did not describe the degree of purification of these three isoenzymes with respect to the original homogenate or, indeed, pursue their further purification. However, the properties of these isoenzymes were investigated in considerable detail. Isoenzymes I and III had pH optima near 5.5, whereas isoenzyme II had a pH optimum in the vicinity of pH 4 and resembled isoenzyme P in this respect. Incubation in 0.05 *M* sodium citrate, pH 4.9, for 15 minutes at various temperatures showed complete thermal inactivation of isoenzyme III at 55°C, whereas isoenzyme I was inactivated only to the extent of 45%, and isoenzymes II and P to the extent of 10–20%. The thermal inactivation of this latter pair generally followed an S-shaped curve, with about 50% inactivation occurring at about 60°, and complete inactivation at 65–70°. The inactivation of isoenzyme I was more gradual. The Michaelis constant, K_m, with *p*-nitrophenyl phosphate as substrate was 1 m*M* for isoenzyme III and 7 m*M* for isoenzyme II.

Table 2 shows the extent of inhibition effected by various substances on the activity of the three isoenzymes. Assays were carried out in 0.05 *M* sodium citrate, pH 4.9, at 37°C. The concentration of substrate was 0.0055 *M* *p*-nitrophenyl phosphate; the reaction was allowed to proceed for 15 minutes, then was stopped by the addition of sodium hydroxide. Units were expressed as micromoles of substrate hydrolyzed per minute per milliliter of enzyme solution. It may be seen that *p*-choromercuribenzoate completely inhibited isoenzyme III, while affecting isoenzymes I and II only slightly. The inhibitions by L-(+)-tartrate and fluoride were in the reverse directions.

The pattern of hydrolysis of various substrates by these three isoenzymes also showed marked differences. When the velocity of hydrolysis of *p*-nitrophenyl phosphate was arbitrarily set at 100, the rates for isoenzyme I were α-naphthyl phosphate, 59; pyridoxine 5-phosphate, 40.

TABLE 2
INHIBITION OF ISOENZYMES OF HUMAN PLACENTAL PHOSPHATASE[a]

Compound	Concentration (mM)	Inhibition of Isoenzyme I (%)	Inhibition of Isoenzyme II (%)	Inhibition of Isoenzyme III (%)
p-Chloromercuribenzoate	0.001	14	7	100
L-(+)-Tartrate	20	41	90	0
Fluoride	50	51	23	5
Pyridoxine	50	11	35	58
Pyridoxine 5-phosphate	10	9	27	67

[a] Based on data of DiPietro and Zengerle (D13).

The corresponding rates for isoenzyme II were 50 and 66, and those for isoenzyme III were 7 and <2. The velocity of hydrolysis of other phosphate esters, such as glucose 6-phosphate or glucose 1-phosphate were, in general, low or negligible. An interesting property of isoenzyme III, not shared by either I or II, was the stimulation of its action on *p*-nitrophenyl phosphate by various purines. For example, 5 mM adenine increased the activity by 83%, and 0.1 mM N^6-benzyladenine or N^6-methyladenine by 35% and 34%, respectively. 6-Ethylmercaptopurine at a concentration of 1 mM had a stimulatory effect of 168%.

4. Intracellular Distribution of Acid Phosphatase

4.1. INTRODUCTION

Since normal human tissues are with rare exceptions (R3) seldom available in amounts adequate for study of intracellular distribution of acid phosphatase or other enzymes, most of our information in this area must be based on investigations in animals. In 1951, while engaged in studying the specific glucose-6-phosphatase of rat liver, Berthet and de Duve (B14) noted that a large proportion of the acid phosphatase in this tissue was apparently linked with the mitochondria. However, further investigations (A12, A13) showed that this binding involved, not the mitochondria as such, but rather cytoplasmic granules which were recovered mainly with the mitochondria and, to some extent also, with microsomes. Indeed, the acid phosphatase appeared to be present in a specific "saclike" structure, the permeability and integrity of which could be altered. Decreasing the concentration of sucrose which had been used in the centrifugal separation of the fraction containing acid phosphatase or even homogenizing this fraction in distilled water resulted in consider-

able increases of the velocity of action of the enzyme on the substrate, sodium β-glycerophosphate.

The acid phosphatase appeared to be associated in the saclike structure with other hydrolytic enzymes, such as β-glucuronidase and cathepsin, which also acted optimally at acid pH levels. Further studies were undertaken to isolate this structure (A13, G2). By means of a differential centrifugation procedure which will be described in detail later, de Duve and his associates (D9, D10) determined the intracellular distribution of total and free acid phosphatase activity and of other enzymes as well. The mean values, expressed as percent of total acid phosphatase activity, were: nuclear, 3.6; mitochondrial, 24.1; light mitochondrial, 40.7; microsomal, 20.1; final supernatant, 13.3.

Several other rat liver acid hydrolases, such as ribonuclease, deoxyribonuclease, cathepsin, and β-glucuronidase, showed a similar intracellular distribution, with a preponderance of activity in the light mitochondrial fraction, in the light and heavy mitochondrial fractions, or in the light mitochondrial and microsomal fractions. The similar pattern of distribution, of these enzymes and acid phosphatase, led de Duve and his associates to the provisional conclusion that these belonged to granules of the same class. For practical purposes, it was proposed to refer to these granules as lysosomes, thus calling attention to their richness in hydrolytic enzymes (D10).

Using electron microscopy, Novikoff *et al.* (N6) found that rat liver fractions rich in these "lysosomal" enzymes, particularly acid phosphatase, showed the presence of mitochondria but had a predominance of single-membrane-limited bodies which were generally electron dense. Fractions with a low acid phosphatase activity rarely showed dense bodies. This observation provided some correlation between the biochemical concept of "lysosomes" and the existence of a structural unit within the cell (S28).

The question arises whether acid phosphatase is present only in lysosomes or whether it exists also in other organelles of the cell (B4). We have just noted that centrifugal methods used by de Duve and his associates (D10) showed a preponderance in, but not a complete confinement of, the acid phosphatase to one ultracentrifugal fraction. The Gomori method (G7) for the definition of acid phosphatase activity, particularly when combined with electron microscopy (N5), has also failed to show exclusive localization in one organelle. It is possible that such staining may be, and indeed has been, regarded as an enzyme-dependent artifact, resulting from a diffusion of reaction intermediates, reaction product, or enzyme (D1).

4.2. Intracellular Distribution of Acid Phosphatase in Liver

Of several methods that are potentially available for determining the intracellular distribution of acid phosphatase and other enzymes, the chief ones currently in use are ultracentrifugal separation and histochemical examination. Each of these has its disadvantages and advantages, some of which have already been indicated. At this point we will consider the quantitative ultracentrifugal methods.

After considerable preliminary work, de Duve *et al.* (D10) proposed the following procedure for rat liver acid phosphatase. Albino adult rats, fasted for 12 hours, were killed by a blow on the head and bled. The liver was quickly taken out, immersed in an ice cold medium, weighed, cut, and dispersed with 3 volumes of 0.25 *M* sucrose in a homogenizer of the Potter-Elvehjem type. After a single run upward against the rapidly rotating pestle, the resulting slurry was centrifuged in the cold at 10,000*g*-min. The sediment, which contained nuclei and unbroken cells, was rehomogenized and centrifuged by 6000*g*-min twice, and the final sediment made up to a volume equal to 4 times the weight of tissue processed, yielding the 1:4 nuclear fraction. The supernatants were combined and made up to volume to form the 1:10 cytoplasmic extract, which was further fractionated. Three particulate fractions were successively isolated by integrated forces of 33,000*g*-min, 250,000*g*-min and 3,000,000*g*-min. The washed granules were finally taken up in small volumes of 0.25 *M* sucrose. The "free" acid phosphatase activity was determined on the various fractions by incubation for 10 minutes at 37°C in the presence of the substrate, β-glycerophosphatase, and of sufficient sucrose to make the concentration of this sugar 0.25 *M* in the mixture. Total acid phosphatase activity was measured in a similar manner on preparations, usually diluted 10-fold in distilled water and exposed for 3 minutes to the action of the Waring Blendor. Care was taken to avoid overheating by the blender, and sucrose was added to a 0.25 *M* concentration in the final assay mixture. The distribution of total and free acid phosphatase activity in various subcellular fractions was determined in 19 experiments, and the mean values for the total acid phosphatase in the fractions, expressed as percent of the total acid phosphatase activity in the cell, were: nuclear, 3.6; heavy mitochondrial, 24.1; light mitochondrial, 40.7; microsomal, 20.1; final supernatant, 13.3.

As may be seen, the light mitochondrial fraction, as obtained by de Duve *et al.* (D10), contained only 40.7% of the acid phosphatase of the rat liver. Starting with this fraction, Sawant *et al.* (S4) attempted

to isolate the lysosomal entity and determine its properties. Male Sprague-Dawley rats were sacrificed by decapitation, and the livers were washed free of blood with cold 0.25 *M* sucrose. All subsequent operations were performed at 0–4°. The livers were homogenized in 0.25 *M* sucrose (1:8, w/v) for 30 seconds in a Waring Blendor at top speed. The pH was adjusted to 7.2 with 5 *N* KOH, and the homogenate was then filtered through muslin.

The homogenate was centrifuged at 750*g* for 10 minutes; the pellet was discarded, and the supernatant was then centrifuged at 300*g* for 10 minutes. The resulting pellet contained "heavy" mitochondria and was discarded, whereas the supernatant was then centrifuged at 16,300*g* for 20 minutes. The supernatant was now discarded, and the resulting pellet which contained what de Duve and his associates had originally termed light mitochondria (D10) was designated as fraction FI. This fraction, FI, as well as fractions FII and FIII, were collected by means of a GSA rotor (r = 16.3 cm) which had compartments for 250-ml bottles. Acid phosphatase in fraction I had 4.2-fold the specific activity of the enzyme in the original homogenate. The degree of purification of other lysosomal enzymes, namely, aryl sulfatase, ribonuclease, and cathepsin were of the same order of magnitude.

The pellet representing FI was resuspended in 0.3 *M* sucrose and centrifuged at 9500*g* for 10 minutes. The supernatant was discarded, and the pellet, designated as FII, was essentially the light mitochondrial fraction. It was resuspended in 0.45 *M* sucrose, layered over a discontinuous gradient of 0.7 *M* sucrose (bottom layer) and 0.6 *M* sucrose (middle layer) and centrifuged at 9500*g* for 30 min. The supernatant was discarded, and the pellet (FIII) was resuspended in 0.7 *M* sucrose.

At this point, the specific activities of lysosomal enzymes in the suspended pellet with respect to the specific activities in the original homogenate as 1 were as follows: acid phosphatase, 23.0; aryl sulfatase, 55; ribonuclease, 20.5; cathepsin, 18. The suspension was centrifuged at 5900*g* for 30 minutes. The pellet was discarded, and the supernatant was carefully decanted. This was centrifuged at 17,000*g* for 20 minutes. The resulting pellet was again washed with 0.7 *M* sucrose, resuspended, and centrifuged with a SS-34 rotor (r = 10.6 cm) in 50 ml tubes to yield the final fraction, FIV. The acid phosphatase activity of this pellet was 5499 nmoles/minute per milligram of N as compared with an activity of 82 nmoles/minutes per milligram of N for the original homogenate. This represented a 67-fold purification; the degrees of purification for other lysosomal enzymes were: aryl sulfatase, 200; ribonuclease, 72; cathepsin, 62. The measurement of the activities of succinoxidase, uricase, and glucose-6-phosphatase as representing the presence of mitochondria,

peroxisomes, or microsomes, respectively, indicated the absence of the first two types of particles in fraction IV and only a 6–7% contamination by microsomes.

The yield of acid phosphatase was 11%, as compared with 30% for aryl sulfatase and 15% for ribonuclease. Obviously, in the attempt to obtain pure preparations of intracellular components, it is inevitable that losses be encountered. Such procedures are therefore not suited for obtaining an estimate of the quantitative distribution of intracellular components. Approximately 5–9% of the lysosomal enzymes—aryl sulfatase, acid phosphatase, and ribonuclease—were present in the free form. The remaining 91–95% of the activities of these enzymes were in the latent form and required alteration of the permeability or disruption of the lysosomal membrane to become active.

It has been estimated that approximately 70–80% of the acid phosphatase in rat liver can be recovered in the lysosomal fraction, the remainder being distributed between the soluble fraction and other subcellular fractions (D9, S15). The question therefore arises concerning the extent to which this remainder is derived from lysosomes broken during the fractionation procedure or whether some acid phosphatase is actually localized in subcellular structures, other than lysosomes.

Shibko and Tappel (S15) approached this problem by comparing the properties of the acid phosphatase in the lysosomal fraction isolated in the manner just described with the properties in the mitochondrial, microsomal, and soluble fractions. Of eleven substrates tested, only F-1,6-diP, AMP, and *p*-nitrophenyl phosphate were hydrolyzed at rates approaching that, or greater than that, of β-glycerophosphate. Some of these are shown in Table 3. The microsomal fraction hydrolyzed glucose 6-phosphate very readily, such hydrolysis obviously reflecting the presence of glucose-6-phosphatase, the characteristic enzyme of this fraction. Although the activities in the soluble fraction were about 1–2% of those in the lysosomal fraction, the relative actions on the various substrates were essentially the same.

The lysosomal and the soluble fractions of acid phosphatase were compared in several other ways. The K_m values (mM) for the lysosomal fractions on various substrates were: β-glycerophosphate, 1.6; fructose 1,6-diphosphate, 2.0; *p*-nitrophenyl phosphate, 1.6; AMP, 0.43; they were not significantly different from those obtained with the soluble fraction. The pH-activity curves with these substrates were similar for the two fractions. Inhibition of phosphatase activity of the lysosomal and soluble fractions occurred at approximately the same concentration of fluoride or L-(+)-tartrate when β-glycerophosphate, AMP, or fructose 1,6-diphosphate were used as substrates. However, with *p*-nitrophenyl phosphate

TABLE 3
SUBSTRATE SPECIFICITY OF ACID PHOSPHATASE IN VARIOUS SUBCELLULAR FRACTIONS OF RAT LIVER[a,b]

Substrate	Fraction			
	Mitochondrial	Lysosomal	Microsomal	Soluble
β-Glycerophosphate	61	4550	425	46
p-Nitrophenyl phosphate	78	6000	520	95
Glucose 6-phosphate	51	640	4100	10
Fructose 1,6-diphosphate	64	3860	840	29

[a] Based on data of Shibko and Tappel (S15).

[b] The activities, expressed as nmoles/mg N/min, were determined from the hydrolysis of 0.05 *M* substrate in 0.1 *M* acetate buffer (pH 5.4) plus suitably diluted subcellular fraction in a total volume of 1 ml during a 15-minute period at 37°.

as substrate, the acid phosphatase was relatively insensitive. For example, at 0.02 *M* inhibitor, lysosomal acid phosphatase was inhibited to the extent of about 95% by L-(+)-tartrate and about 80% by fluoride; the inhibitions of the soluble acid phosphatase were about 25% and 40%, respectively.

The electrophoretic patterns of the soluble fraction and of a mixture of the soluble and lysosomal fractions were essentially the same. The acid phosphatase was located in two regions: a distinct band migrating toward the anode, and a less distinct area that moved toward the anode. Chromatography on DEAE-cellulose yielded 3 peaks for each fraction. Relatively larger amounts of fluoride and L-(+)-tartrate-insensitive acid phosphatase were present in the soluble fraction.

That the properties of lysosomal acid phosphatase do not change upon solubilization was evident when the lysosomal fraction was subjected to alternate freezing and thawing for 10 times and then centrifuged at 100,000*g* for 1 hour; 50% of its acid phosphatase was released into the suspending medium, and the remainder was associated with the lysosomal membrane and precipitable. The patterns of hydrolysis of various substrates with the exception of G-6-P which reflected membrane-bound glucose-6-phosphatase, were the same for the two subfractions.

These results indicate that the major portion of the soluble acid phosphatase is similar to that of the lysosomal phosphatase and may be derived from the injury or breaking of these particles. On the other hand, the presence of a phosphoprotein phosphatase in the soluble fraction and its absence from the lysosomes, and the existence also of a fluoride and an L-(+)-tartrate-insensitive acid phosphatase in the soluble fraction

indicate intracellular sources of acid phosphatase, other than lysosomes.

When histochemical techniques for acid phosphatase were carried out at the electron microscopic level (N5, N6), dense bodies about 0.4 μ in diameter having a single outer membrane were observed in intact cells of the liver. In addition bodies with similar morphological characteristics were found to be located along the fine bile canaliculi. Indeed lysosomes as a whole showed considerable polymorphism, apparently the result of their association with the different materials that are phagocytized by the cell (S29).

4.3. Intracellular Distribution of Acid Phosphatase in Other Tissues

4.3.1. *Introduction*

Most of our knowledge of lysosomes arises from studies of these particles in rat liver. These studies have also supplied considerable evidence that, by virtue of its more than a dozen hydrolytic enzymes, the lysosome can play a role in digesting material foreign to the cell, its own cell, or that its enzymes may be discharged outside the cell to produce lytic effects. It is also possible, as has been shown for rat liver (R1), that lysosomes may be heterogeneous in terms of their enzyme contents. We shall now examine the extent to which acid phosphatase is distributed intracellularly in tissues other than the liver and in species other than the rat.

An approximate idea of such a distribution is available from the study of Shibko *et al.* (S17). These investigators made a 20% homogenate of washed tissue in 0.25 *M* sucrose containing 1 m*M* EDTA. This homogenate was divided into two fractions as follows. A preliminary centrifugation at 750*g* for 10 minutes (7500*g*-min) removed unbroken cells and nuclei. The supernatant solution was filtered through glass fiber and recentrifuged at 75,000*g* for 45 minutes (3,375,000*g*-min). The resulting pellet was suspended in 0.25 *M* sucrose; on the basis of considerations that have already been presented, this suspension would consist of mitochondria, lysosomes, and most of the microsomes. Shibko *et al.* (S17) analyzed it for various hydrolytic enzymes, including acid phosphatase. Obviously, designating these enzymes as "lysosomal" is based on the assumption that negligible amounts of these enzymes are present in the other organelles, such as mitochondria and microsomes, that are centrifuged down between 7500*g*-min and 3,375,000*g*-min.

With this understanding in mind, the values of Shibko *et al.* (S17) may be presented. For livers of various species the total acid phosphatase activity, expressed as nanomoles of substrate hydrolyzed per milligram

of N per minute, were: rat, 455; sheep, 26; hog, 15; ox, 12. The fraction, centrifuged down by 750*g* for 10 minutes, had the following acid phosphatase activities: sheep, 39; hog, 22; ox 20. These values would apparently constitute a substantial portion of the acid phosphatase in some of the species, but it is difficult to conclude from the study by Shibko *et al.* (S17) that these represent acid phosphatase in the nuclei or in the unbroken cells.

For the spleens of various tissues, the 7500*g*-min and 3,375,000*g*-min fractions had the following activities, respectively, again expressed as nanomoles per milligram of N per minute: ox, 116 and 54; hog, 162 and 0; sheep, 50 and 10. It would, therefore, appear that in spite of unbroken cells being centrifuged down at 7500*g*-min a substantial portion of the acid phosphatase would appear to reside in or attach to the splenic nuclei.

Histochemical techniques have indicated that acid phosphatase need not be confined to the lysosomes. For example, with β-glycerophosphate as substrate, this enzyme has been found to be present in the small vesicles in the Golgi region, in vesicles without apparent relationship to the Golgi region and in other elements resembling cisternae of smooth endoplasmic reticulum of cells of rat kidney adenomas (S14), and in the Golgi region of the rat anterior pituitary (S25). Kalina and Bubis (K1) observed that the acid phosphatase activity in small neurons of rat spinal cord appeared as granules, i.e., lysosomes, whereas the activity in large neurons was localized both in scattered granules and in a network of filaments, representing rough endoplasmic reticulum. Fixation in glutaraldehyde for 30 minutes or in formaldehyde for 4 hours abolished the acid phosphatase activity in the large neurons, but did not affect that in the small cells. Inhibitors like sodium fluoride also showed differences between the large and small cells, and indicated the possible existence of two types of acid phosphatase acting on β-glycerophosphate. Maggi (M1) has reviewed other instances in which acid phosphatase acting on β-glycerophosphate is located in intracellular components other than lysosomes.

4.3.2. *Rat Heart*

Employing as a criterion for lysosomal localization the activity of hydrolases in the fraction centrifuged down between 750*g* for 10 minutes and 75,000*g* for 45 minutes and treated with the detergent X-100, Shibko *et al.* (S17) found no evidence of acid phosphatase in the lysosomal moiety or indeed in the homogenate as a whole of pigeon heart muscle. Romeo *et al.* (R8) subjected a mitochondrial suspension of beef heart tissue to isopycnic centrifugation in sucrose–water solutions and isolated a particulate fraction ($d = 1.174$) that showed a high concentration of

latent hydrolytic enzymes and a comparatively low concentration of cytochrome *c* oxidase and appeared to have the main features of a lysosomal fraction. β-Galactosidase, β-glucuronidase, cathepsin, acid ribonuclease, and acid deoxyribonuclease were present. No mention was made, however, of the presence of acid phosphatase.

Maggi (M2) undertook to reexamine the relationship between the number of lysosomes and the degree of acid phosphatase activity in the rat heart. After homogenization of the minced heart tissue in 0.28 *M* sucrose–EDTA, pH 7.2, the homogenate was centrifuged at 2400 rpm for 10 minutes to get rid of unbroken cells, nuclei, and some heavy mitochondria. The supernatant was then centrifuged at 20,000 rpm for 30 minutes in a Spinco Model L. The pellet was resuspended in sucrose–EDTA and frozen and thawed six times before acid phosphatase activity was assayed. With *p*-nitrophenyl phosphate as substrate, the activity of the supernatant was 2243 nmoles of *p*-nitrophenol liberated per hour per milligram protein and was four times the activity of the pellet. The pH optimum lay between 5.6 and 5.8. This acid phosphatase activity was not affected appreciably by the chlorides of Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, and only partially inhibited by up to 100 m*M* sodium fluoride. When β-glycerophosphate was used as substrate and the pellet treated in the same manner, no enzyme activity could be detected in the supernatant, whereas the activity of the pellet was 77 nmoles of P liberated per hour per milligram of protein. This activity was greatly stimulated by 10 m*M* Mg^{2+} and was inhibited completely by 10 m*M* sodium fluoride. The pH optimum lay between 4.8 and 5.0. These results would indicate that rat heart contains more than one acid phosphatase, possibly in different subcellular compartments.

4.3.3. *Pancreas* (*Mouse*)

The distribution of acid phosphatase in the mouse pancreas cell was studied by Van Lancker and Holtzer (V2) by means of centrifugal methods. In general, the pancreas was homogenized at 0° for 3 minutes in 0.25 *M* sucrose with the Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenate was diluted to a concentration of 1 g of tissue in 5 ml of total suspension. The suspension was then subjected to successive centrifugations: each pellet was homogenized and washed once and the washing was added to the supernatant. Triton X-100 was added to the assay mixture to assure complete liberation of acid phosphatase. The distribution as percent of the total was as follows: nuclei ($6 \times 10^3 g$-min) 5.0; zymogen granules ($8.4 \times 10^3 g$-min) 5.7; large and small mitochondria (16.8×10^3 to $263 \times 10^3 g$-min) 29.8; microsomes (1585×10^3 to $3170 \times 10^3 g$-min) 19.8; postmicrosomes ($6340 \times 10^3 g$-min) 7.1; supernatant, 19.2.

The centrifugal method of separation employed by Van Lancker and Holtzer (V2) was among the earlier ones in the field, and there was probably considerable cross contamination of the fractions. Nonetheless, the distribution seems more disperse than that obtained by de Duve *et al.* (D10) for rat liver with a comparable method. For example, in the case of the mouse pancreas the small mitochondrial fractions, c, d, and e, obtained by centrifugation between 17×10^3 and $263 \times 10^3 g$-min contained 27% of the acid phosphatase and the succeeding "microsomal" fractions, f and g, obtained by centrifugations between $263 \times 10^3 g$-min and $3170 \times 10^3 g$-min, contained 24% of the acid phosphatase (V2). For rat liver, comparable fractions, obtained by centrifugation between 33×10^3 to $250 \times 10^3 g$-min and 250×10^3 to $3000 \times 10^3 g$-min contained 41 and 20%, respectively (D10).

4.3.4. *Kidney (Rat)*

Roche and Baudoin (R7) first noted that the kidney contained acid phosphatase. Employing homogenates of rat kidney in 0.45 *M* sucrose and centrifugations in the same concentration of sucrose, Shibko and Tappel (S16) defined four subcellular fractions as follows: unbroken cells and nuclei, 2.5 minutes at 650*g*; lysosomal-mitochondrial fraction, 1 minute at 10,000*g*; mitochondrial-microsomal fraction, 20 minutes at 12,000*g*; microsomal fraction, 60 minutes at 10,000*g*; soluble fraction, that remaining as supernatant from the last centrifugation. The enzyme activities of the subcellular fractions were measured after freezing and thawing 10 times. With sodium β-glycerophosphate as substrate, the activities of acid phosphatase expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein, were: homogenate, 53; lysosomal fraction, 847; mitochondrial fraction, 212, microsomal, 74; soluble fraction, 16. With *p*-nitrophenyl phosphate as substrate, the activities, again expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein, were 52, 507, 146, 82, and 40, respectively.

It may thus be seen that the lysosomal fraction defined by Shibko and Tappel (S16) as that centrifuging down in 0.45 *M* sucrose for 1 minute at 10,000*g* was the most active. However, a more active fraction could be isolated by methods similar to that employed by Sawant *et al.* (S4) for rat liver. After removal of the nuclei and unbroken cells, the supernatant was filtered through glass wool and centrifuged for 5 minutes at 5900*g*. The pellet consisted of three well-defined layers. The upper pinkish and the middle buff-colored layers were removed, and the lower dark brown layer was suspended in 0.6 *M* sucrose and centrifuged for 5 minutes at 5900*g*. Any light-colored material on the surface of the pellet was removed, and the lower pellet was suspended in 0.6 *M* sucrose. This suspen-

sion was contaminated to some extent with mitochondrial and microsomal fractions, as manifested by determination of marker enzyme activities, but electron microscopic examination showed about 95% of intact lysosomes and about 5% of mitochondria. Microsomal fragments were seldom visible.

Employing the procedure of de Duve *et al.* (D10) for determining intracellular fractions by centrifugation in 0.25 *M* sucrose, Wattiaux-de Coninck *et al.* (W2) obtained the following percentage distribution for kidney: nuclear fraction, 20.9; heavy mitochondrial, 33.9; light mitochondrial, 7.6; microsomal, 19.9; final supernatant, 15.3. The sum of the acid phosphatase activities in the light mitochondrial and microsomal fractions, in which it is presumed that most of the lysosomes should be gathered, was 27.5%, as compared with 60.8% obtained by the same method for liver (D10). The absolute total acid phosphatase activity for all fractions of the kidney was 5.53 units/g and that for liver was 6.06 units/g (D10).

4.3.5. *Prostate (Bull, Rat)*

In man and in other mammalian species, the major mass of the prostate, usually consisting of the right and left lateral and the middle lobes, is composed of alveoli lined with columnar epithelium embedded in a thick fibromuscular stroma. These alveoli constantly secrete a fluid which is drained off by a system of branching ducts that empty into the floor and lateral surfaces of the posterior urethra. The normal secretion is dependent upon the degree of androgenic stimulation and amounts to about 0.5–2 ml per day. The prostatic secretion, which is characterized by very high acid phosphatase activity, is a milky fluid which contains citric acid, choline, cephalin, cholesterol, proteins, and electrolytes similar to those found in the plasma.

Attempts to determine the intracellular distribution of acid phosphatase in the prostate must take into account the presence of this enzyme in the extracellular secretion. Employing centrifugal methods, Siebert *et al.* (S20) found that of the total acid phosphatase present in bull prostate homogenate, 0.7% was in the nuclear fraction, 41% in the mitochondrial fraction which presumably included the lysosomal component, and 84% in the microsomal and supernatant components. The finding that the sum of these activities exceeded that in the homogenate was considered to represent removal of inhibitors during separation of the fractions.

The intracellular distribution of acid phosphatase in the ventral prostate of the rat has also been investigated by Bertini and Brandes (B15). Groups of male rats of two weight levels, 350 ± 20 g and 180 ± 10 g

were fasted overnight and sacrificed by exsanguination. The ventral prostates were homogenized in three times their weight of ice-cold 0.35 *M* sucrose, 0.001 *M* EDTA. The pellet resulting from centrifugation at 600*g* for 10 minutes represented the nuclear fraction, N. The supernatant, cytoplasmic fraction, C, was centrifuged at 10,000*g* for 3 minutes to yield the mitochondrial fraction, M; a light mitochondrial fraction, L, was obtained by centrifugation at 41,000*g* for 6 minutes and 40 seconds; a microsomal fraction, P, was obtained by centrifugation at 105,000*g* for 30 minutes. The resulting supernatant was designated S. Total enzyme activity was elicited by freezing and thawing 7 times in an acetone–dry ice mixture. Sodium β-glycerophosphate was used as the substrate for determination of total and free acid phosphatase activity. In 350-g rats, the distribution of this enzyme activity, expressed as percent of the sum of the activities in the nuclear and cytoplasmic fractions, was: nuclear 11.3; mitochondrial, 21.8; light mitochondrial, 19.8; microsomal, 10.5; supernatant, 35.1, with a recovery of 99.8%. No substantial difference was found for the 180-g rats, except for a slight increase of latent acid phosphatase.

The free acid phosphatase activities expressed as percentage of the total acid phosphatase activity for each of the fractions were: nuclear, 53; mitochondrial, 33; light mitochondrial, 55; microsomal, 127; supernatant, 101. The sum of the free activities of all fractions was 71% of the total activity. The comparable values for the fractionation of rat liver (D10) were: nuclear, 60; mitochondrial, 18; light mitochondrial, 12; microsomal, 50; supernatant, 106. The sum of the free activities of all fractions was 22% of the total activity. Thus the latent activity of acid phosphatase in the prostate was less than in the liver.

Pellets separated from nuclei-free homogenates in 0.25 *M* sucrose by centrifugation at 45,000*g* for 6 minutes and washed twice by resuspending in excess 0.25 *M* sucrose were analyzed by isopycnic gradient centrifugation. The sucrose concentrations ranged in density from 1.20 to 1.145. The activity curves for acid phosphatase and cytochrome oxidase and the concentration curve for protein showed essentially the same distribution, with a peak at a density of about 1.180. These results as well as those on the latency of acid phosphatase indicate the possibility that the lysosomes containing the enzyme become disrupted during fractionation or during isopycnic gradient centrifugation, and that acid phosphatase may be absorbed to other subcellular particles in a nonspecific manner.

Electron microscopic studies of rat prostatic epithelium, showed acid phosphatase to be present largely in localized areas of the cytoplasmic matrix, lacking in many cases structures which would be comparable

with that of the "sacs" having a single-layered, lipid membrane, such as had been described in liver parenchymal cells (B29, B30, H1, H4). Koenig (K10) has raised the question whether the lysosomal enzymes may not, at least in some tissues, be conjugated ionically with acidic glycolipids in a solid complex.

4.3.6. *Testis and Semen*

The intracellular distribution of acid phosphatase in the testis has only recently been studied (R9). The following fractions were obtained from a homogenate of the testes of adult Swiss mice in 0.25 *M* sucrose: nuclear, 600*g* for 5 minutes; heavy mitochondrial, 10,300*g* for 3 minutes; light mitochondrial, 41,000*g* for 7 minutes; microsomal, 105,000*g* for 30 minutes; supernatant of the last centrifugal procedure. This supernatant fraction contained 65% of the free, uninactivated acid phosphatase, whereas the light mitochondrial fraction, usually presumed to include the lysosomes, contained only 19% of the free acid phosphatase. Activation was accomplished by freezing and thawing of the testicular tissue five times. The activity of the homogenate increased from 6.6 to 9.2 μg P liberated per minute per milligram of protein. As was to be expected, no activation was apparent in the supernatant fraction, but that of the light mitochondrial fraction increased from an activity of 1.27 to one of 3.49 μg P liberated per minute per milligram protein, and constituted 38% of the postactivation total acid phosphatase activity.

The character of the intracellular distribution of acid phosphatase in the testis appears to differ from that in the kidney or liver, where a major portion of the enzyme is in the lysosomal fraction. Although the finding in the testis may represent a difference in cellular organization, the possibility also exists that the acid phosphatase may be less firmly bound to the lysosomal structure in the testis than in the liver and may be more readily solubilized during the process of homogenization.

As long ago as 1935, Kutscher and Wolbergs (K12) observed that semen and the prostate are among the richest sources of acid phosphatase in the human body. In a more recent survey (B11) the acid phosphatase activities of seminal plasma in various species, determined as milligrams of nitrophenol liberated by 100 ml seminal plasma from 0.006 *M* *p*-nitrophenyl phosphate, in 60 minutes at 37°C and pH 4.9 were: human, 274,000; cock, 15,000; turkey, 4000; bull, 570; rabbit 85. Human seminal plasma is made up by the secretory fluids produced in the epididymides, vasa deferentia, ampullae, seminal vesicles, the prostate and the bulbourethral (Cowper's) and urethral (Littre's) glands (M4). The semen contains many particulate bodies. Best known, of course, are the spermatozoa, which are formed in the seminiferous

tubules of the testis and remain in the epididymis for a "ripening" period before ejaculation.

Dingle and Dott (D12) have noted that certain lysosomal enzymes, including acid phosphatase, in bull and ram semen are sequestered into membrane-limited droplets, which are shed from the spermatozoon during maturation but persist in the seminal plasma. In bull semen, these droplets were separated and found to contain acid phosphatase, β-glucuronidase, acid protease and ribonuclease whereas other lysosomal enzymes, such as β-*N*-acetyl glucosaminodase, β-galactosidase, and hyaluronidase were present predominantly in the spermatozoa. Dott and Dingle (D14) have determined the total, free and bound acid phosphatase of whole semen. Bull semen was diluted 1:20 with buffered saline, and a sample was treated with detergent and the total acid phosphatase activity determined with *p*-nitrophenyl phosphate as substrate. The activity was 3510 units (μg nitrophenol liberated per hour per milliliter of semen). The remainder of the semen was sedimented at 1000*g* for 5 minutes and then at 10,000*g* for 10 minutes. The combined activity of the pellets, after washing, was 750 units or 21% of the total and represented the bound activity, that is, the activity in the cells and droplets. The enzyme activity in the 10,000*g* supernatant represented the free or nonsedimentable enzyme (2760 units). In the ram, the values were quite different: 6500 units for the total, 1600 units for the free or nonsedimentable acid phosphatase and 4900 units or 75% of the total activity for the bound enzyme in the cells and droplets.

The cytoplasmic droplet in bull and ram serum is spherical with a diameter of approximately 3 μ. When viewed by phase contrast microscopy, it has a dark "granulated" region; electron microscopy reveals that this region consists of vesicles and membranous or tubular structures. The concentration of acid phosphatase in droplets is 4.3 μg nitrophenol liberated per hour per 10^6 particles, much higher than the activity, 0.2 μg nitrophenol liberated per hour per 10^6 spermatozoa. Ribonuclease, acid protease, β-glucuronidase showed similar ratios. Dott and Dingle (D14) submitted additional information which indicated that in the bull and, to a lesser extent in the ram, the lysosomal enzymes cease to be associated with the spermatozoon during its maturation.

4.3.7. *Leukocytes and Macrophages*

Peritoneal exudates, containing large numbers of polymorphonuclear leukocytes, may be produced in rabbits by the intraperitoneal injection of 200 ml of 0.1% glycogen (C7). Leukocytes from the exudate were withdrawn 4 hours later, washed once in cold 0.34 *M* sucrose, and

then lysed in the same medium. The sucrose lysate was separated into three fractions by differential centrifugation: the nuclear pellet at 400*g* for 10 minutes; the granule pellet at 8200*g* for 15 minutes; the resulting supernatant. Acid phosphatase as well as several other enzymes, alkaline phosphatase, nucleotidase, ribonuclease, deoxyribonuclease, and β-glucuronidase, were predominantly localized in the granule fraction. The characteristics of acid phosphatase in leukocytes will be discussed further in Section 6.9.

In a subsequent study, Cohn and Wiener (C8) considered the particulate hydrolases of macrophages. Peritoneal macrophages were induced in rabbits by intraperitoneal injection of oil, and aveolar macrophages were obtained from the lungs of normal rabbits or of rabbits following intravenous injection of BCG. The cells were centrifuged, washed, and assayed. The acid phosphatase activity, expressed as micrograms of P liberated from sodium β-glycerophosphate per hour at 38° by 10^6 macrophages was: 2.6 for oil-induced peritoneal macrophages, 20.7 for normal alveolar macrophages, and 37.0 for BCG-induced alveolar macrophages. The corresponding activities per milligram of N were 118, 600, and 1073. Differential centrifugation of homogenized oil-induced macrophages showed that the acid phosphatase, as well as other hydrolases, was localized as follows: about 20% in the nuclear fraction (500*g* for 12 minutes), approximately 65% in the fraction sedimented by centrifugation at 12,000*g* for 15 minutes, and the remainder, about 10–15%, in the supernatant fraction. Most of the acid phosphatase activity as well as that of the other hydrolases was latent, as repeated freezing and thawing or a longer time period for assay increased the activities greatly.

4.4. Digestive Function of Lysosomes

The importance of lysosomes in physiopathological autolysis, intracellular digestion and engulfing processes was pointed out by de Duve (D7, D8) and Novikoff (N5). Within recent years several studies have appeared in which the actions of purified lysosomal preparations on proteins, carbohydrates and lipids have been considered (A14, C5, M3, S2).

Sawant *et al.* (S2) studied the digestion of rat liver homogenate, mitochondria, microsomes, and nuclei by a purified preparation of lysosomes. A few values, particularly for inorganic phosphate, may be quoted to show the extent of digestion at 37°C and pH 7.0. With regard to the action on liver homogenate, 1 mg of lysosomal protein formed 28 and 38 nmoles of amino acids and peptides in 0.5 hour and 3.0 hours,

respectively. Under the same conditions 380 and 840 nmoles of inorganic phosphate were formed in these periods. Similar results were obtained by the action of lysosomes on isolated mitochondria and microsomes. Negligible effects were obtained for the action on nuclei at pH 5.0. Degradation of mitochondria was influenced by the concentration of lysosomes, pH, and temperature of the reaction system.

The formation of inorganic phosphate was, of course, a manifestation of phosphatase action. In this connection, two features were of interest. First, in the mitochondrial degradation by lysosomes, the rate of formation of inorganic phosphate decreased from values of about 2 μmoles in 30 minutes at pH of 4–5 to a value of about 0.6 μmole in 30 minutes at pH 6.0, then rose again to about 2 units at pH's of 7 to 9. Second, tartrate and fluoride were potent inhibitors; $2.5 \times 10^{-4}\,M$ tartrate decreased the rate of release to 21% of the control value, and $1 \times 10^{-3}\,M$ inhibited the formation completely. Fluoride had a similar effect.

5. Polymorphism of Acid Phosphatase in Human Erythrocytes

5.1. Introduction

We have already discussed the properties of human erythrocytic acid phosphatase (Section 3.3), and we pointed out that, like acid phosphate in other tissues, it may exist in several isoenzymatic forms. In 1963, Hopkinson *et al.* (H13) subjected hemolysates of human red cells from an English population to horizontal starch-gel electrophoresis for 17 hours at 5°C. The gels were then sliced horizontally, covered with 0.05 M phenolphthalein sodium diphosphate at pH 6.0, and allowed to incubate for 3 hours at 37°C. Five different electrophoretic patterns of acid phosphatase activity could be distinguished in different individuals. Shortly thereafter Lai and his associates (L2) confirmed these findings and discovered an additional sixth pattern which had been predicted by Hopkinson *et al.* (H13). The distribution of these patterns in various types of population was assiduously pursued within the next several years, and several new ones were discovered in Negro populations (G3, K2).

It is of interest that within several years after the observations of Hopkinson *et al.* (H13), other human erythrocytic enzymes such as phosphoglucomutase, glucose 6-phosphate dehydrogenase, phosphogluconate dehydrogenase, adenylate kinase, peptidase, and adenosine deaminase were explored intensively with respect to their polymorphism (H2, H11). However, we shall concern ourselves here only with acid phosphatase.

5.2. Electrophoresis

The starch gel electrophoretic patterns obtained by Hopkinson (H11) and his associates (H14) and subsequently by others in Harris' group (H2) and elsewhere in the world (G3, K2) are shown in Fig. 1. Initial studies by Hopkinson *et al.* (H13) on 139 randomly selected English males and females revealed the existence of five patterns with the following frequencies in the population: type A, 10.1%; type BA, 46%; type B, 34.5%; type CA, 36%; type CB, 5.8%. On the basis of genetic considerations, they predicted the existence of a sixth type, C. Lai (L1) and his associates (L2) obtained a different distribution in a Brazilian population, but reported evidence for the existence of the type C electrophoretic pattern. Additional studies by Hopkinson (H11) modified the incidences of the patterns slightly: A, 13%; BA, 43%; B, 36%; CA, 3%; CB, 5%; C, 0.016%.

In their original studies, Hopkinson *et al.* (H13) had employed a 0.0025 *M* succinic acid–0.0046 *M* Tris buffer, pH 6.0 for their gel preparations and a 0.041 *M* citric acid/NaOH buffer, pH 6.0, as a bridge solution. Using vertical electrophoresis, a mixture of 10.2 ml of formic acid (90%) and 9.25 g of NaOH per liter as the bridge buffer, pH 5.0, and a 1:10 dilution of this to make up the gels, Giblett and Scott (G3) discovered a new electrophoretic pattern, designated as RA, in the red cell hemolysate of a Seattle Negro woman. This and two related patterns, RB and RC, were characterized by a pair of relatively fast moving components together with either typical A or B or C zones.

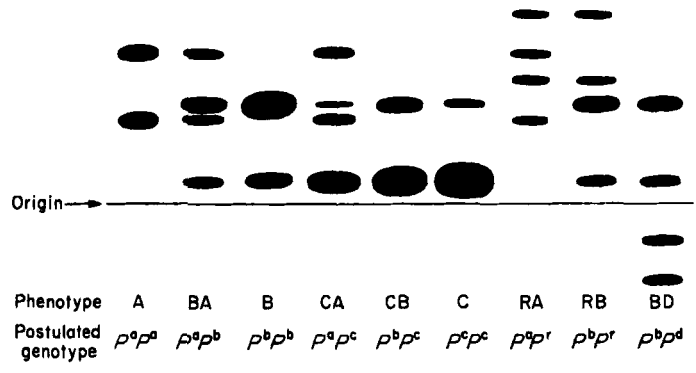

Fig. 1. Diagram of electrophoretic patterns of the several red cell acid phosphatase phenotypes. After Hopkinson (H11).

Still another electrophoretic pattern, BD, was observed in a Texan Negro (K2).

5.3. Genetics

These different electrophoretic patterns or phenotypes reflected the possible existence of alleles, or contrasting genes situated at the same locus in homologous chromosomes. Study of the acid phosphatase patterns or phenotypes in 440 families and in their 925 offspring indicated that the various phenotypes were determined by three alleles, P^a, P^b, and P^c and that phenotypes A, B, and C have the homozygous genotypes P^aP^a, P^bP^b, and P^cP^c, respectively. Similarly, phenotypes BA, CA, and CB corresponded to the heterozygotes P^aP^b, P^aP^c, and P^bP^c, respectively (H11). The distributions of phenotypes observed in the children were not significantly different from the expected Mendelian proportions. For example, in the data presented by Hopkinson (H11) and his associates, there were 94 matings of parents, each of whom had patterns of BA and therefore had the heterozygotic genotype, P^aP^b. Of the 185 children, one-fourth, or 46, should have had the phenotype A, one-half, or 92, should have had the phenotype BA, and one-fourth, or 46, the phenotype B. The incidences actually found were 40, 91, and 54, respectively.

Gene or allele frequencies may be derived from the distribution of various phenotypes among a number of individuals. For example, in a study of 1010 individuals in England, the gene frequencies for acid phosphatase were: P^a, 0.36; P^b, 0.59; P^c, 0.05. Population data from other countries have accumulated rapidly since Hopkinson's original studies, and some of these may be noted briefly (Table 4). It may be seen that the gene frequencies for the United States for persons of European origin were essentially the same as those in England (G3). For Australians (L1) and South Africans (H11) of European origin, the gene frequencies of P^a were 0.33 and 0.32, respectively, somewhat lower than those for England and the United States; the gene frequencies of P^b were somewhat higher, 0.64 and 0.62, respectively. In Negro populations, regardless of their geographic location, the incidence of P^a was strikingly lower and that of P^b was correspondingly higher (B31, G3, H11, K2). The most extreme deviation from average gene frequencies was observed in a group of 140 Tristan Da Cunhan islanders with a P^a value of 0.09 and a P^b value of 0.91. The Alaskan Eskimos and Atabascan Indians show unusually high values for the frequency of the P^a gene, 0.56 and 0.67, respectively (S11).

Recent studies have amplified the essential features of the genetic studies that we have just considered. Wyslouchowa (W12) found the

TABLE 4
GENE FREQUENCIES UNDERLYING RED CELL ACID PHOSPHATASE POLYMORPHISM IN VARIOUS POPULATIONS[a]

Geographical location	Number of individuals tested	Gene frequencies			
		P^a	P^b	P^c	Pr
England	1010	0.36	0.59	0.05	—
U.S.A.: European origin	193	0.39	0.55	0.06	—
U.S.A.: Negro, Ann Arbor	224	0.17	0.82	0.01	—
U.S.A.: Negro, Seattle	363	0.23	0.76	0.015	0.010
Australia: European origin	260	0.33	0.64	0.03	—
New Guinea: Trobriand	484	0.20	0.79	0.01	—
South Africa: European origin	99	0.32	0.62	0.06	—
South Africa: Cape Colored	174	0.28	0.70	0.02	—
Nigeria: Yoruba	129	0.17	0.83	—	—

[a] Based on review of data by Hopkinson (H11).

following gene frequencies in a group of 1064 Poles: P^a. 0.319; P^b, 0.585; P^c, 0.096. These are not essentially different from those reported for English and other European populations (H11). In his study of Danish populations Lamm (L3) discovered a family with the rare P^d allele which had hitherto been observed only in Negro families (G3, K2). Herbich *et al.* (H6) studied the pedigree of a family in which unusual segregation of the acid phosphatase phenotypes occurred. In generation II, the father (II. 7), a heterozygous type CA, and the mother (II. 8), a homozygous type B, had a child (III. 1) with a normal type C pattern, indicating a P^c allele. In other words, the mother did not appear to have transmitted a P^b allele to her son. Again when the parents (I. 8 and I. 9) of the mother (II. 8) were tested, her father had a type A phenotype and the mother a type BA phenotype. Thus here, too, the father did not appear to have transmitted the expected allele, P^a, to his offspring, and the type B pattern exhibited by II. 8 was presumably determined by a P^b allele transmitted to her from the heterozygous mother, I. 9. These and other findings in this pedigree suggested that there existed in this family a rare "silent" acid phosphatase allele, P^o, such that P^aP^o individuals were phenotypically A, P^bP^o individuals phenotypically B, and P^cP^o individuals phenotypically C.

An interesting role of acid phosphatase alleles on an unusual condition in man has recently been reported by Bottini *et al.* (B27). It has long been appreciated that subjects with erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency may have a severe hemolytic crisis after ingestion of fava beans. Although this deficiency is a necessary condition for the occurrence of hemolytic episodes, not all, indeed only

about 30% of subjects with G-6-PD deficiency, exhibit clinical favism. Bottini *et al.* (B27) observed that the frequencies of P^a and P^c alleles of the gene for erythrocytic acid phosphatase in a group of Roman males with favism were 0.355 and 0.109, respectively, and were significantly higher than the corresponding frequencies, 0.261 and 0.080, in a group of normal Roman males. A similar relationship held for Sardinian males with favism, but not for females with this condition. This observation indicates that alleles of a gene coding for an enzyme polymorphic in all human populations affect the fitness of the involved phenotypes in special genotype (G-6-PD deficiency) and nongenotypic conditions (ingestion of fava beans).

5.4. Quantitative Distribution

The phenotypes of erythrocyte acid phosphatase not only exhibit differences in electrophoretic behavior, but also show variation in total acid phosphatase activity. Spencer *et al.* (S26) studied the distribution of red cell phosphatase activities in hemolysates from 275 individuals with various phenotypes. The assay was performed with 0.01 *M* disodium *p*-nitrophenyl phosphate as substrate at pH 6.0 in citrate buffer. The units of activity were expressed as μmoles of *p*-nitrophenol liberated in 0.5 hour at 37°C per gram of hemoglobin. These results are shown in Table 5.

If it is assumed that all the acid phosphatase activity observed in the various types is determined by the three genes, P^a, P^b, and P^c then the question arises whether the quantitative effects of these genes are additive in a simple way. It may be seen from Table 5 that half the mean activity, 122.4, in type A plus half the mean activity, 188.3, in type B is equal to 61.2 + 94.2 or 155.4. This value is in good agreement with 153.9, the mean value actually observed for type BA. Other equations

TABLE 5
Acid Phosphatase Activity in Various Phenotypes[a]

Phenotype	Number of individuals	Mean activity[b] (units)	Standard deviation (units)
A	33	122.4	16.8
BA	124	153.9	17.3
B	81	188.3	19.5
CA	11	183.8	19.8
CB	26	212.3	23.1

[a] Based on data of Spencer *et al.* (S26).

[b] Expressed as micromoles of *p*-nitrophenol liberated in 0.5 hour at 37°C per gram of hemoglobin.

based on the additive hypothesis give calculated values in accord with those actually observed. It is thus possible to assign activity values to the various alleles: 61 for P^a; 94 for P^b; 120 for P^c. Hopkinson (H11) subsequently enlarged the series to 336 individuals, but the results were essentially the same as those that have just been described.

5.5. Biochemical Characteristics of Phenotypes

Differences in the electrophoretic patterns and activities between the phenotypes raised the possibility that they might also be characterized in a more specific and detailed biochemical manner. In 1949, Abul-Fadl and King (A4) observed that 0.5% formaldehyde inhibited erythrocyte acid phosphatase completely, whereas 0.01 *M* L-(+)-tartrate had no effect. When 0.5% formaldehyde was added to the reaction mixture in gel electrophoresis (H13), it completely inhibited all variants of erythrocyte acid phosphatase, so that no zones of activity in any of the five types were visible after the 3-hour incubation.

Scott (S10) and later Luffman and Harris (L13) failed to find any kinetic differences between the variants. Scott (S10) purified the acid phosphatases from two homozygous, phenotypically different, human red cells. These were designated as AA and BB and corresponded to A and B, respectively, in the terminology of Hopkinson *et al.* (H13). Employing several different substrates and sodium acetate as buffer, with a final reaction pH of 6.5, Scott (S10) failed to find any differences between the Michaelis constants of these two isozymes; with phenyl phosphate as buffer, the K_m values were 0.87 and 0.75 m*M* for the AA and BB phenotypes, respectively. With α-glycerophosphate as substrate, the corresponding K_m values were 5.8 and 5.5 m*M*. Nor were any differences observed when *p*-nitrophenyl or phenolphthalein diphosphate were the substrates. A few small differences could be elicited. Thus, phosphate inhibited the AA variant more than the BB variant. The maximum velocity of the AA variant was relatively lower at lower pH. However, these findings did not account for the finding that the AA variant had 65% of the activity of the BB variant.

Luffman and Harris (L13) applied several other criteria in an attempt to differentiate among the various phenotypes. Incubation of hemolysates representing these phenotypes showed that at temperatures of 47°C to 52°C types CA and CB were denatured more slowly than the other types tested, A, BA, and B. For example, after 30 minutes at 50°C, the average losses in activity were: 52% for CA and 57% for CB, as compared with losses of 89% for A, 83% for BA, and 80% for B.

Incubation with guanidine or urea at 28°C for 20 minutes showed denaturation to be dependent on the concentration, but there were no

differences in the rate of denaturation between these phenotypes. Each of the variants exhibited substantial phosphotransferase activity. For example, in the presence of 20% methanol, the phosphotransferase activity was approximately 300% of the hydrolytic activity in the absence of methanol, but again the phosphotransferase activity was essentially the same for all the phenotypes tested. The rates of hydrolysis of 14 different phosphate esters as substrates were determined, and although the patterns relative to the rate of hydrolysis of *p*-nitrophenyl phosphate were of interest, there appeared to be no significant differences between the patterns for the various acid phosphatase types.

Samples of the four variants, A, BA, B, and CB were subjected to gel filtration together with several substances of known molecular weights as markers. In every instance, all the acid phosphatase activity emerged from the column as a single peak subsequent to the elution of myoglobin (molecular weight of about 17,000) and cytochrome *c* (molecular weight of about 12,400). This finding suggested that erythrocytic acid phosphatase may have a very low molecular weight in the region of 7000–10,000. There were no differences in the elution positions of the enzyme in the different phenotypes (L13).

The column chromatography of the five common phenotypes were also studied by Hopkinson and Harris (H12). A 10-ml aliquot of the supernatant from a centrifuged hemolysate was applied to the DEAE column which had been washed with Tris-phosphate buffer (pH 8.0). The column was then washed with the starting buffer to elute the hemoglobin. The enzyme was eluted with an exponential gradient of sodium chloride in Tris-phosphate buffer and collected in 2-ml fractions at a flow rate of 20 ml/hour.

Two distinct peaks of acid phosphatase activity were detected in each phenotype, but the positions of these peaks differed. For example, in phenotype A, the peaks were approximately at tubes 150 and 190; in B, at about 130, 170, and 265; in BA, at 110 and 155. In these three, the first peaks showed minor enzyme activity. In CA, there was a major peak at about tube 130 and a smaller one at about tube 170. The shape of the curves varied according to the phenotype tested. In general, these results confirmed what gel electrophoresis originally showed, namely, that there are charge differences between the various isoenzymes. The electrophoretic patterns may also be influenced by the type of buffer used to make up the starch gel (K2).

5.6. Polymorphism in Other Tissues

The question arises whether polymorphism can be demonstrated for the acid phosphatases of other human tissues. Beckman and Beckman

(B10) carried out starch gel electrophoretic studies in 1200 individual placentas and in extracts of seven different organs obtained at autopsy from 14 individuals. The tissues showed different combinations of one or more bands of four distinct and, in some respects, biochemically different, acid phosphatase components. These were designated as A, B, C, and D in order of decreasing anodal mobilities. For example, in the case of heart tissue one individual showed three bands, ABD, whereas the remaining 13 showed a combination of two bands, BD. With regard to kidney tissue, 13 individuals had a combination of ABD, and one individual had a pair, BD.

The C component was present in all extracts of the 1200 placentas, but in none of the other organs. The typical placental combination was BCD. Three out of 1000 placentas showed a deviating electrophoretic pattern. The B zone was not affected. In addition to the usual C component, there was another somewhat slower component. The D zone contained three components of which the fastest one coincided with the normal D band. This deviating pattern in the three placentas probably represented genetically determined variants.

6. Alterations of Serum Acid Phosphatase Activity in Disease

6.1. Introduction

At the beginning of this review, we noted that acid phosphatase activity was first found to be present in human urine (D11). Approximately ten years later, Kutscher and his associates (K11, K12, K13) described its presence and properties in the various organs of the male genital tract and in other tissues. The clinical significance of this enzyme in prostatic disease was elicited by Gutman and his associates (G11, G12, G13, R6, S30). Subsequent investigators not only extended these findings, but also sought to correlate alterations in serum acid phosphatase activity with treatment of various types, to determine whether alterations in nonprostatic disease might also occur and to examine basic mechanisms involved in these alterations. Since different methods were employed in these various studies, we shall describe the results of these studies in some detail.

6.2. Normal Values for Serum Acid Phosphatase Activity

The method of Gutman and Gutman (G10, G14), an application of the King-Armstrong (K5) method for alkaline phosphatase to acid phosphatase, was described earlier in this paper. The activity by this method was defined in units, as the number of milligrams of phenol liberated in 1 hour at 37°C by 100 ml of serum. These have been fre-

TABLE 6
VALUES FOR SERUM ACID PHOSPHATASE IN NORMAL ADULT MALES BY THE METHOD OF GUTMAN AND GUTMAN (G10, G14) OR SLIGHT MODIFICATIONS OF IT

Author	Number of individuals	K.A. units[a]		
		Range	Mean	Standard deviation
Gutman and Gutman (G10)	10	0.6–2.0	1.2	0.39
Sullivan *et al.* (S30)	30	<3.0	—	—
Fishman and Lerner (F1)	13	0.7–1.7	1.2	0.34
Fishman *et al.* (F2)	104	<5.0	1.8	0.8
Day *et al.* (D6)	136 (20–39 years old)	1.1–4.7	2.70	0.57
Day *et al.* (D6)	179 (40–79 years old)	0.8–4.0	2.47	0.56
Benotti *et al.* (B12)	22	—	1.2	1.2

[a] Units are equal to milligrams of phenol liberated from phenyl phosphate in 1 hour at 37°C by 100 ml of serum.

quently referred to as King-Armstrong (K.A.) units. Table 6 shows various series of values obtained with this method in normal males. Values on large groups of patients with nonprostatic disease have not been included. Some of these results were obtained in connection with the proposal of a procedure for the determination of the "prostatic" component of acid phosphatase in serum by the use of the inhibitor, L-(+)-tartrate (F1). We shall discuss this aspect in greater detail later.

The determination of the range of acid phosphatase activity in apparently healthy normal males must naturally take into account the effect that benign prostatic hypertrophy may have. This problem was considered by the writer and his associates in 1956 (D6). Utilizing the Gutman procedure (G10, G14), together with the modifications Fishman and Lerner (F1) had proposed, the "total" and "prostatic" moieties of acid phosphatase were determined in a large group of male individuals who had presented themselves at a cancer-prevention clinic for general examination. Of a group of 141 men from 20 to 39 years of age, five had some degree of prostatic enlargement. The remaining 136 had serum acid phosphatase activities ranging from 1.1 to 4.7 units with a mean value of 2.70 K.A. units and a standard deviation of 0.57 unit (Table 6). In this group, there were four men with acid phosphatase values higher than 3.8. In the five individuals who had clinically abnormal prostates but no evidence of prostatic carcinoma, the serum acid phosphatase activities were all normal, this is, less than 3.84 units (2.70

plus two standard deviations). In a group of 179 males, 40–79 years of age, with clinically normal prostates, the serum acid phosphatase activities ranged from 0.8 to 4.0 K.A. units and averaged 2.47 K.A. ± 0.56 K.A. units. There were five individuals with acid phosphatase values greater than 3.8 K.A. units. In this same age group, there were 46 males who had enlarged prostates but only four of these had values greater than 3.84 units: 4.1, 4.2, 4.2, and 4.7 units. Thus it would appear that in this total group of 366 males, neither age nor benign prostatic enlargement had an effect on acid phosphatase activity; 96% of the values were less than 2 standard deviations above the mean, and the remaining individuals had acid phosphatase values between 2 and 3 standard deviations above the mean value.

The mean normal values listed in Table 6 are not, in general, different statistically from each other. The value of 2.7 ± 0.57 (D6) appears significantly higher than that calculated for the group of 13 subjects reported by Fishman and Lerner (F1), 1.2 ± 0.34 K.A. units, even though Fishman and his co-workers stated elsewhere (F2) that normal values ranged from 0.5 to 5.0 units. It is difficult to state whether these differences among different series are due to variations in the nature of the population or in methodological differences, such as spontaneous hydrolysis in the substrate (B12) or the loss in acid phosphatase activity that a serum undergoes when it is separated from the clot and allowed to remain at room temperature for varying periods of time before analysis (W7).

The next most common method for determination of serum acid phosphatase activity was based on the use of sodium β-glycerophosphate as substrate. This method and its modifications have been described in Section 2.3. Values obtained by these methods in normal males are shown in Table 7; a liberal summary of these values indicates a mean value of about 0.4 ± 0.2 Bodansky units.

6.3. Serum Acid Phosphatase in Carcinoma of the Prostate

Carcinoma of the prostate is today one of the three most frequent causes of death from neoplastic disease in men in the United States (G4). The early studies of Gutman and his associates (G11, G12, R6, S30) established that serum acid phosphatase activity was elevated very frequently in patients with metastatic carcinoma of the prostate. It is of interest to consider briefly the uncertainties inherent in the development of this relationship. Skeletal metastases, if sufficiently large, are of course detectable by roentgenographic examination, but smaller ones may not be, and metastases to soft tissues may likewise be undetectable. For example, in 15 cases of metastazing carcinoma reported by Gutman

TABLE 7
VALUES FOR SERUM ACID PHOSPHATASE IN NORMAL MALES BY THE METHOD OF A. BODANSKY (J2) OR SLIGHT MODIFICATIONS THEREOF

Investigator	Number of normals	Range	Mean	Standard deviation	Upper limit of (mean + 2 SD)
Bodansky (B19)	43	—	0.19	0.048	0.29
Shinowara *et al.* (S18)	20	0.1–1.1	—	—	1.1
Woodard (W8, W9)	47	0.11–0.88	0.45	—	0.88
Marshall and Amador (M5)	36	0.1–0.5	0.28	0.12	0.51

and Gutman (G11) in 1938, 12, or 80%, exceeded the normal range of serum acid phosphatase activity, 0.5–2.5 K.A. units, as previously defined by them. The highest was 516 K.A. units, and the lowest was 3.1 K.A. units. But the latter patient, who had extensive osteoplastic metastases, had already been subjected to resection of the prostate. Of the three patients with values of 1.6, 1.5, and 1.5 units, two had questionable or no bony metastases and the third patient had osteolytic and osteoplastic metastases but had been treated by implantation of radium seeds.

Shortly thereafter, a second paper from the Gutman group (R6) presented a series of 28 determinations of serum acid phosphatase on 19 patients with roentgenographic evidence of skeletal metastases. These ranged from 1.6 to 260 K.A. units; 23, or 89%, were higher than 3.0 K.A. units, the upper limit of normal, as defined in this study. Thirteen patients with prostatic carcinoma but without any roentgenographic evidence of skeletal metastases had serum acid phosphatase levels of 0.5 to 2.6 K.A. units, all less than the upper limit of 3.0 K.A. units of Sullivan *et al.* (S30). These findings in Gutman's group are summed up most comprehensively in Table 8. It may be seen that 85% of patients with prostatic carcinoma and skeletal metastases had elevations above their designated upper limit of normal, 3.0 K.A. units. Five patients had sensationally high values, more than 1000 K.A. units. The frequency of elevations in the patients with no bone metastases, as visualized roentgenographically, was 11%. This group was not described in detail, and it is possible that some of the patients had soft tissue metastases or nondetectable bone metastases. The general presumption is that in these cases extracapsular extension of the prostatic carcinoma had not proceeded too far. Sullivan *et al.* (S30), however, described one

TABLE 8
ELEVATIONS OF SERUM ACID PHOSPHATASE ACTIVITY IN DISEASES OF THE PROSTATE

Disease	Sullivan *et al.* (S30)		Herbert (H5)	
	No. of patients	Incidence of elevations (%)	No. of patients	Incidence of elevations (%)
Carcinoma of prostate with bone metastases	130	85	35	89
Carcinoma of prostate without bone metastases	70	11	47	42
Benign prostatic hypertrophy	75	0.0	95	9.5
Prostatitis	10	0.0	—	—

patient who died of congestive heart failure 3 days after a normal serum acid phosphatase determination of 2.4 K.A. units. Autopsy showed an early, regionally invasive but not distantly invasive carcinoma of the prostate. Table 8 indicates that benign prostatic hypertrophy or prostatitis does not cause any elevations of acid phosphatase. This confirmed early studies by Gutman and Gutman (G11).

Reports by others, using the Gutman method (G10, G14), did not quite agree with the preceding results. Thus Herbert (H5), utilizing a normal range of 1 to 4 K.A. units, observed a similar incidence of serum acid phosphatase elevations in prostatic carcinoma with skeletal metastases, 31 out of 35 patients, or 89%, but a much higher incidence of elevations than Gutman's group in prostatic carcinoma without bone metastases, namely, 20 of 47 patients, or 42%. Four of these patients had substantial elevations, that is, above 10 K.A. units. Herbert (H5) also observed a 9.5% incidence of elevations, slight though these were, in benign hypertrophy of the prostate.

There are a number of other studies in the literature in which serum acid phosphatase was determined by the method of A. Bodansky or slight modifications thereof. The important feature of this method is the use of sodium β-glycerophosphate as substrate. As has been noted earlier, Woodard (W8) established the normal range in 20 normal females as averaging 0.38 unit (milligrams of phosphorus liberated in 1 hour by 100 ml of serum under certain standardized conditions) and ranging from 0.06 to 0.89 unit. The activity in 47 normal males was essentially the same—an average of 0.45 unit and a range of 0.11 to 0.88 unit. Patients with various diseases not involving the bladder, rectum, or prostate had essentially the same average values and range, so that a

broad range of "normal" values could be established for 492 patients without prostatic diseases as averaging 0.39 unit, with a range of 0.00 to 0.98 unit.

The data of Woodard and Dean (W11) and of Woodard (W8) for patients with prostatic disease are sufficiently comprehensive to represent several other studies based on the use of Na β-glycerophosphate instead of phenyl phosphate as substrate. Thus, in 107 patients with carcinoma of the prostate with metastatic lesions to the bones, the range of serum acid phosphatase activities was 0.10 to 520 Bodansky units, with 74% above the upper limit, 0.98 unit. In 51 patients where the tumor was locally invasive, the range was 0.0 to 12.9 units, with 31, or 60%, above the upper limit of normal. In 20 patients with carcinoma confined to the prostate, the range was 0.28 to 5.9 units with 5, or 25%, above the upper limit of normal. In 9 patients with metastases to distant soft parts, the range was 0.72–18.3 units with 78% above the upper limit of normal. Twelve patients with prostatitis and 10 patients with carcinoma of other origins involving the prostate showed serum acid phosphatase values well within the normal range.

On the ingenious assumption that early bone metastases might reveal themselves by increased elevations of acid phosphatase in the bone marrow blood, Chua *et al.* (C3) determined the enzyme activity in 10-ml samples of blood taken simultaneously from a cubital vein and from the area of the posterior-superior iliac spine. The Bodansky method (B17, J2) with β-glycerophosphate as substrate was employed to determine the acid phosphatase activity in the serums from both sites. Four of 12 patients with clinically localized carcinoma of the prostate had elevated bone marrow acid phosphatase with normal serum acid phosphatase levels and skeletal surveys negative for metastases. One of these patients had a strikingly elevated bone marrow acid phosphatase (116 Bodansky units), and a bone marrow biopsy which disclosed the presence of metastases even though there was no radiological evidence of these.

Of 13 patients with clinical extension of the cancer beyond the prostatic capsule, seven who were receiving antiandrogens and had undergone bilateral orchiectomy showed normal bone marrow and serum acid phosphatase activities. Of the remaining six patients, one had slightly elevated bone marrow acid phosphatase activity and a normal serum acid phosphatase activity. In the other five patients, both the marrow and the serum acid phosphatase values were elevated, but the former were higher. Of 13 patients who had osteoblastic changes in the skeletal system, increased acid phosphatase values were obtained in both bone marrow and serum in 10 cases, but the values in the bone marrow

samples were much higher. The results in these three groups indicate that measurement of bone marrow acid phosphatase may offer aid in the early diagnosis of bone metastases from prostatic carcinoma.

The importance of defining the normal range before evaluating serum acid phosphatase elevations with prostatic disease is well brought out by the more recent study of Marshall and Amador (M5). Using β-glycerophosphate as substrate and a group of 36 healthy ambulatory males, 41 to 57 years of age, these investigators found a range of 0.1 to 0.5 unit, with a mean of 0.28 $\pm$ 0.116 unit and an upper limit (mean + 2 SD) of 0.51 unit. This range was narrower and the mean value lower than those obtained by Woodard (W8, W10) or by Shinowara *et al.* (S18), and was much closer to that, 0.19 $\pm$ 0.048, obtained by A. Bodansky (B18). Employing their own criterion for the normal range, Marshall and Amador (M5) found that 27 of 57 patients, or 46%, with intracapsular prostatic carcinoma and 15 of 27, or 56%, of patients with soft tissue metastases had values above 0.5 unit, the upper limit of normal. However, if Shinowara and his associates' (S18) value of 1.1 unit had been taken as the upper limit of normal, the incidence of elevations in patients with carcinoma of the prostate and skeletal metastases, soft tissue invasion, or intracapsular confinement would have decreased to 65%, 30% and 16%, respectively.

6.4. Specificity of Serum Acid Phosphatase Determination for Carcinoma of the Prostate

6.4.1. *Kinetic Considerations*

Preceding data have already indicated, and subsequent discussion will confirm, the view that the use of β-glycerophosphate as substrate yields a lower incidence of elevations in nonprostatic disease than the use of phenyl phosphate and in this sense constitutes a more specific method. Several authors have distinguished between these two methods by using the terms "acid glycerophosphatase" and "acid phenylphosphatase" activities (B6, T7). This specificity might be explained by the fact that sodium β-glycerophosphate, the substrate in the Bodansky procedure, is hydrolyzed more readily than phenyl phosphate, the substrate in the Gutman procedure, by acid phosphatase from the prostate and that the converse situation holds for enzyme derived from other tissue sources, such as the erythrocyte or the liver. Abul-Fadl and King (A4) found that in the presence of 0.01 Mg^{2+}, the rate of hydrolysis of 0.02 M β-glycerophosphate was 30 mg P per 30 minutes per 100 ml of human prostate extract and was much higher than the rate of hydrolysis, 0.3 mg of P by human erythrocytes. In contrast, phenyl phosphate was

hydrolyzed much more readily by erythrocytic acid phosphate, 48 mg of P per 30 minutes per 100 ml, a rate essentially equal to the rate of hydrolysis by prostate.

The Michaelis constant, K_m, for prostatic phosphatase is much higher with β-glycerophosphate than with phenyl phosphate as substrate. Nigam *et al.* (N3), employing a purified preparation of human prostate phosphatase, obtained Michaelis constants of 0.75 mM with acetate buffer (pH 5.1) for phenyl phosphate and 4.0 mM for glycerophosphate and corresponding values of 0.09 mM and 2.0 mM with citrate buffer at pH 4.9. Tsuboi and Hudson (T3) found values for K_m of 0.15 mM for phenyl phosphate and 2.4 mM for β-glycerophosphate for a 300-fold purified human prostatic preparation, although these investigators noted that the degree of purification had little effect on the value of K_m.

In addition to the studies cited above, there are several others showing that phenyl phosphate is much more readily hydrolyzed than β-glycerophosphate by acid phosphatase from human erythrocytes, whereas no such marked difference exists with respect to human prostatic phosphatase (B2, T1, T3). Unfortunately, there do not appear to be any systematic investigations of the substrate–velocity relationship for the acid phosphatases of other human tissues. In general, the available data would indicate that β-glycerophosphate is a more specific substrate than phenyl phosphate for the detection and assay of acid phosphatase coming from the prostate.

6.4.2. *Inhibition by* L-(+)-*Tartrate*

Another procedure to increase the specificity of acid phosphatase determinations for prostatic disease has involved the use of L-(+)-tartrate to distinguish between the enzyme from the prostate and other tissues. In a series of papers from 1947 to 1949, Abul-Fadl and King (A1, A2, A3, A4) studied the properties of various acid phosphatases and reported that 0.01 M L-(+)-tartrate inhibited the hydrolysis of phenyl phosphate by human prostatic acid phosphatase dissolved in normal saline or in plasma to the extent of 95%, but had no effect on the hydrolysis by acid phosphatase from erythrocytes. The inhibitions of acid phosphatases from other human tissues were as follows: liver, 70%; kidney, 80%; spleen, 70%.

Several years later, Fishman and his associates (F1, F2, F3) applied this principle to the determination of the tartrate-inhibitable or prostatic fraction in serum. This method involved the hydrolysis of disodium phenyl phosphate into phenol and phosphate by serum in the absence and presence of 0.02 M L-(+)-tartrate for a period of 1 hour at 37°C. Suitable blank and control solutions were employed. The activity in the

absence of L-(+)-tartrate represented the "total" acid phosphatase activity. The activity in the presence of 0.02 *M* L-(+)-tartrate was subtracted from the "total" acid phosphatase to denote the inhibited or "prostatic" acid phosphatase. The values for "prostatic" phosphatase in normal subjects ranged from zero to 0.6 K.A. unit (F2). The proposed diagnostic utility of this procedure was based on the assumption that early cases of prostatic carcinoma might have normal values for the total acid phosphatase, but would reveal elevations of the prostatic fraction.

In the series of 13 normal males studied by Fishman and Lerner (F1) to which reference has already been made and in which the serum total acid phosphatase ranged from 0.7 to 1.7 K.A. units (Table 6), the prostatic acid phosphatase component ranged from 0.1 to 0.3 K.A. The following distribution of prostatic acid phosphatase activities was obtained in a series of 151 male patients without prostatic cancer but suffering from other diseases, such as cardiovascular disorders, other forms of cancer, arthritis, and diabetes: 0 K.A. unit, 4%; 0.1 unit, 37%; 0.2 unit, 37%; 0.3 unit, 13%; 0.4 unit, 7%; 0.5 unit, 2%. The distribution of the corresponding total acid phosphatase activities in this group was: 0.0–0.5 unit, 13.0%; 0.6–1.0 unit, 43%; 1.1–1.5 units, 35%; 1.6–2.0 units, 6%; 2.1–3.0 units, 3% (F1). In a group of approximately 100 female patients with the same diseases, the distribution of the prostatic component was understandably shifted to the lower values. Thus 65% of the patients had prostatic acid phosphatase levels of 0.1 unit or less. The corresponding distribution of total acid phosphatase activity was 0–0.5 unit, 7%; 0.6–1.0 unit, 33%; 1.1–1.5 units, 39%; 1.6–2.0 units, 14%; 2.1–3.0 units, 9%.

Fishman *et al.* (F3) reported that in a group of 12 patients with proven prostatic cancer, five patients without evidence of metastases had normal values for total acid phosphatase between 1.3 and 3.0 K.A. units. Yet in four of these the prostatic portion was 0.7 to 2.2 units, all elevated above the normal level of 0.5 unit. These investigators also indicated that early elevations of the prostatic component in the presence of a normal total acid phosphatase might be the herald of progression of disease with ultimate elevations of total acid phosphatase.

As was previously noted (Section 6.2), the total and prostatic serum acid phosphatase levels were determined by the method of Fishman and Lerner (F1) in a series of 365 males attending a cancer-prevention clinic (D6). The values for the total acid phosphatase activities in the 315 patients of this group who had no prostatic enlargement have already been described (Table 6). In groups of the size under consideration, values within 2.5 standard deviations of the mean can be considered as normal;

accordingly, for the group 20–39 years of age, the upper limit of normal for the total acid phosphatase would be 4.13 units and that for the prostatic acid phosphatase would be 0.65 K.A. units, respectively. The corresponding upper limits for the group, 40–79 years of age were essentially the same, namely, 3.87 and 0.66 K.A. units, respectively.

Table 9 shows the number of abnormal total and prostatic acid phosphatase values in male individuals who had presented themselves at a cancer clinic and who on examination showed no benign prostatic hypertrophy (D6). Because of the possibility that, in accordance with Fishman and his associates' concept (F3), these abnormal values for the prostatic portion might be a forerunner of advancing disease, determinations were repeated after various intervals. The individual with an initial prostatic acid phosphatase value of 1.14 units showed a value of 0.36 units 5 weeks later and a value of 0.30 units 6 weeks after his first visit.

Table 9 shows that, in the upper as well as the lower age group of persons with clinically normal prostates, there were essentially no valid elevations in the total or prostatic acid phosphatase. In addition, five individuals in the younger age group (20–39 years) had clinically ab-

TABLE 9

PROSTATIC SERUM ACID PHOSPHATASE LEVELS IN MALE PATIENTS (WITHOUT PROSTATIC ENLARGEMENT) OF A CANCER PREVENTION CLINIC[a]

Age group	Number	Number of normal total and normal prostatic values	Number of high total and normal prostatic values	Number of normal total and high prostatic values	Number of high total and high prostatic values
20–39 years	136[b]	131	2 (4.23/0.06 4.53/0.39)	3 (3.03/0.69 3.09/0.99 3.84/1.14)	0
40–79 years	179[c]	171	3 (3.96/0.39 3.99/0.18 3.99/0.21)	5 (3.09/0.69 3.51/0.75 3.51/0.81 3.54/1.38 3.81/0.75)	0

[a] Based on data of Day *et al.* (D6).

[b] Normal mean values in this group were 2.70 ± 0.57 K.A. units for the total serum acid phosphatase, and 0.14 ± 0.205 K.A. unit for the prostatic moiety. Upper limits of normal were 4.13 and 0.65 K.A. units, respectively.

[c] Normal mean values in the group were 2.47 ± 0.65 K.A. units for the total serum acid phosphatase and 0.15 ± 0.204 K.A. unit for the prostatic moiety. Upper limits of normal were 3.97 and 0.66 K.A. units, respectively. Data according to Day *et al.* (D6).

normal prostates, and four of these had normal values for the total and prostatic acid phosphatases. The fifth had a normal total acid phosphatase value of 3.09 K.A. units, but a value of 1.09 units for the prostatic moiety. In the 224 persons in the older age group, of whom 179 had normal prostates, the incidence of clinically abnormal prostates was much higher, 45, or 26%. Yet 40 of these had normal total and prostatic phosphatases. Of the remaining five, three had high total and normal prostatic acid phosphatases, namely 4.14/0.54, 4.17/0.24 and 4.74/0.18 K.A. units. One had a normal total and high prostatic acid phosphatase value of 3.24/1.02 K.A. units, and the remaining individual had both a high total and high prostatic acid phosphatase value of 4.17/1.98 K.A. units.

There were only eleven in the entire series of 365 patients who had high prostatic acid phosphatase values at their first visit. In each of the patients in this group who had subsequent determinations, the values for the prostatic acid phosphatase were normal. For example, the patient in the older group who had elevated values for the total and for the prostatic fraction, namely, 4.17 and 1.98 units, on the first visit had normal values on the second visit 3 months later, namely, 2.85 and 0.39 units, and normal values of 2.85 and 0.06 units 1 week later.

Clark and Treichler (C4) have studied the psychic factors involved in prostatic secretion, and it is possible that physiological or psychological stimuli might have played a role in yielding high values for prostatic acid phosphatase activity on the first visit. These patients were followed for a period of 6 months to a year and no evidence arose which indicated that these elevations had any pathological significance (D6).

In 1956, Fishman *et al.* (F2) summed up their experience with a series of 91 cases of proven cancer of the prostate and a total of 1198 patients with other diseases. Of these 91 cases, 32, or 35%, had elevated total serum acid phosphatase activities. This incidence was much lower than that, 85%, reported by Sullivan *et al.* (S30) in 1942 or the value of 89% reported by Herbert (H5) in 1946 for patients with carcinoma of the prostate and skeletal metastases. These investigators had used the method of Gutman and Gutman (G10, G14), which was essentially the same method as that employed by Fishman and Lerner (F1) and gave the same ranges of normal values (Table 6). The possibility existed that treated cases had normal total serum phosphatase activities and thus weighted the overall incidence toward a low value. However, the data of Fishman *et al.* (F2) show that the incidence of total serum acid phosphatase activities in treated cases was 20/52, or 38%, even higher than the incidence 12/39, or 31%, in the untreated cases.

Determinations of the prostatic serum acid phosphatase activity in

these 91 cases yielded the following incidences of elevated values: 84% in the group as a whole, 87% in the untreated cases, and 81% in the treated cases. Although these values were much higher than those for the incidence of elevations of total acid phosphatase activity in these cases, they were of the same order of magnitude as those reported for the total acid phosphatase activity by Sullivan *et al.* (S30) and by Herbert (H5).

In the series of 91 cases studied by Fishman *et al.* (F2), the incidence of elevations of total acid phosphatase was 25 of 53 cases or 47% in patients with bone metastases, two of 12, or 16%, in patients with soft tissue metastases and four of 26, or 15%, in patients with no metastases. These values are also much lower than the overall values obtained by Bodansky and Bodansky (B19) in a review of the literature up to 1951, according to which total acid phosphatase was elevated in 81% of 349 cases with bone metastases and in 24% of 218 cases without such metastases.

Whatever may be the reason for the low incidence of elevations of total serum acid phosphatase activity in Fishman and his associates' (F2) series of proven cases of carcinoma, the higher incidence of elevations of prostatic acid phosphatase activity indicates that in the patients of this series the determination of the latter was a more sensitive indicator of the presence of prostatic carcinoma. Moreover, when serum prostatic acid phosphatase activities were determined during the course of a patient's illness, they paralleled the exacerbation or remission of the disease. Whitmore *et al.* (W4) have considered the relationship of clinical status to the total and prostatic acid phosphatase activities in 20 patients with proven carcinoma of the prostate.

It is of interest to note a report in which a patient who had been operated for a gastric malignancy ten years prior to admission began to show symptoms of metastases, especially to the bone, and upon further study revealed high total and prostatic serum acid phosphatase values (S21). The former ranged from 4.7 to 8.1 K.A. units and the latter from 1.8 to 2.3 K.A. units. Various forms of therapy for prostatic carcinoma failed to affect the course of the disease. Postmortem examination yielded neither gross nor microscopic evidence of prostatic carcinoma.

6.4.3. *Adventitial Elevations in Serum Acid Phosphatase Activity*

Massage, palpation, or other trauma or pressure on the prostate may result in sudden elevations of the serum acid phosphatase. Hock and Tessier (H10) observed that prostatic massage caused elevations above the initial value in 17 of 20 patients. The serum acid phosphatase usually

attained its maximal value within 1 hour after massage and returned to normal levels in 24–48 hours. The highest value observed in this series was 15.5 Bodansky units, as compared with the normal range of 0.0 to 0.8 unit. Infarcts of a noncarcinomatous prostate gland have been reported to be associated with high serum acid phosphatase (S27). Daniel and Van Zyl (D2) observed that ordinary digital rectal palpation of 24 patients with cystic benign hypertrophy was followed by a significant rise of serum acid phosphatase in three of the patients, probably as a result of the rupture of wall cysts and the release of phosphatase-rich secretion into the blood. The author has noted elevations in serum acid phosphatase that appeared to be due to the use of catheters or the formation of fecal impactions.

Bonner *et al.* (B26) also studied in some detail the changes in total and prostatic serum acid phosphatase following prostatic massage. Five patients without a prostate gland showed no alteration in either the total or prostatic acid phosphatase during 2 hours after massage. In three patients with carcinoma of the prostate, alterations reflected the severity of the disease. One patient had an initial total acid phosphatase value of 40.0 K.A. units and a prostatic phosphatase activity of 37.0 K.A. units. These rose to 46.0 and 42.0 K.A. units, respectively, 0.5 hour after massage. In a second patient, the total serum acid phosphatase activity rose from 5.4 to 7.4 K.A. units in 0.5 hour, and the prostatic acid phosphatase from 5.2 to 6.4 K.A. units. The third patient in this group had an initial total serum acid phosphatase activity of 1.2 K.A. units and a prostatic fraction of 0.2 K.A. unit. There was no significant rise in either value.

6.5. Factors Involved in Elevation of Serum Acid Phosphatase in Carcinoma of the Prostate

6.5.1. *General Considerations*

The mechanisms involved in maintaining the level of a serum enzyme in the circulation are largely unknown. Generally, it may be conceived that these levels are the dynamic resultant of several processes: (a) the rate of production of the enzyme by one or more tissues; (b) the rate of secretion of the enzyme by the tissues into the circulation; (c) the extent of damage of enzyme-rich tissue with consequent leakage into the circulation; (d) the degradation of the enzyme in the circulation or its degradation by various tissues as the blood circulates through these tissues; (e) the excretion of the enzyme through the kidney, particularly if the enzyme is of a relatively low molecular weight.

6.5.2. *Tissue Acid Phosphatase Activity in Carcinoma of Prostate and in Metastases from Carcinoma of the Prostate*

Although the fibromuscular structure of the prostate has some acid phosphatase activity, histochemical studies reveal that most of the activity is present in the epithelial cells of the secreting glands (R1a). In carcinoma of the prostate, the staining of the epithelial cells for acid phosphatase show greater variation. The staining may be less, equal to, or greater than in the normal prostate (R1a).

Woodard (W8, W9) showed that the carcinomatous prostate contains less acid phosphatase than the normal gland. Specimens from 19 normal prostates yielded a range of 54–1350 units per gram of tissue and an average value of 478 Bodansky units per gram, whereas analysis of 13 carcinomatous prostates from untreated patients gave a range of 16–600 units per gram, and an average of 258 units per gram. The average values for 12 carcinomas from treated patients was 20 units per gram of tissue and for 26 glands with benign hypertrophy, 775 units per gram. In contrast to the decrease of acid phosphatase activity generally found in the carcinomatous prostate, metastatic sites show markedly increased activities, as compared with the normal tissue that is the site of the metastases. For example, Gutman *et al.* (G13) found, in one case, the acid phosphatase activities of osteoblastic metastases were 19.0 K.A. units per gram in the lumbar vertebra and 18.6 units per gram in the rib metastases, as compared with values of 0.1 to 1.3 units per gram at similar normal sites in patients without prostatic carcinoma metastases. Woodard (W9) observed that the primary site as well as metastases in patients with osteogenic sarcoma had low acid phosphatase activities, from 0.09 to 0.85 Bodansky units per gram. In contrast, the activities at metastatic sites in patients with prostatic carcinoma were much higher, frequently about 15 units per gram, and, in one instance, ranged up to 185 units per gram.

6.5.3. *Serum Acid Phosphatase during Treatment of Patients with Carcinoma of Prostate*

In 1941 Huggins and Hodges (H17) observed that treatment of carcinoma of the prostate by bilateral orchiectomy or by estrogen injection resulted in many instances in clinical improvement of the patient and a concomitant decrease of the serum acid phosphatase activity. They also found that in each of eight patients serum acid phosphatase activity fell rapidly after bilateral orchiectomy (H17). For example, in one patient, the serum acid phosphatase decreased from 26 K.A. units immediately preoperatively to 5 K.A. units, slightly above the upper limit of normal,

within a period of 7 days after operation. During a subsequent period of observation for 150 days, the serum acid phosphatase fluctuated between 2.5 to 7.5 units. In a second patient, the serum acid phosphatase decreased from 35 units preoperatively to 3 units within 9 days postoperatively and thereby fluctuated between 3 and 6 units during a subsequent observation period of 104 days.

The injection of estrogen had a similar effect. For example, one patient was injected with 30 mg of stilbestrol in 23 days. The serum acid phosphatase decreased from 48 to 4.5 K.A. units and remained at the latter level for another 10 days of observation. In general, the decreases were not as marked as those reported for patients who had been orchiectomized.

The observations by Huggins and his associates (H17, H20) have been generally confirmed since 1941 (F3, H8, S23, W1). However, there have been reports, particularly in more recent years, that the level of serum acid phosphatase may not always bear a clear relationship to the apparent clinical progress of the disease or the extent of the metastases at autopsy. This lack of relationship is instructive and may be illustrated by the following two cases studied by Bodansky (B22). The first patient, a 70-year-old man, had had a transurethral resection four years previously and had been diagnosed as having prostatic carcinoma. Orchiectomy was performed, and he was placed on stilbestrol therapy. He remained asymptomatic for approximately two and a half years when pain developed in the hip, back, and both rib cages. No record of previous blood biochemical studies was available, and these were now instituted. During the next 5 months, the serum acid phosphatase remained at very high levels, fluctuating between about 80 and 130 Bodansky units (normal 0–0.8 unit). Roentgenographic studies revealed widespread osteoblastic and some osteolytic metastases in the spine, ribs, pelvic bones, left femur. Hypophysectomy was performed and the patient was given cortisone therapy and followed closely until his death some 3 months later. The alkaline phosphatase was less than 10 Bodansky units on many repeated occasions and decreased toward a normal level at about a month and a half preceding his death. These relatively low levels indicated absence of any sizable intrahepatic or osteoblastic skeletal metastases. Until the patient's death, the serum acid phosphatase continued to oscillate between 90 and 130 Bodansky units. It would appear that during the 5 months of study there was no growth or extension of metastases, but rather an active production by these metastases of large amounts of acid phosphatase.

A second patient, P.G., 76 years of age, was found in April 1952 to have an enlarged prostate, but only a slightly elevated serum acid phos-

phate of 3.1 Bodansky units. The patient refused operation and by August had developed osteoblastic metastases. Bilateral orchiectomy was performed, and a bladder biopsy confirmed the presence of prostatic carcinoma. During the next year and a half, he received in succession radiation therapy, estrogen and various types of steroid therapy. Although there was clinical and roentgenographic evidence of the progress of the disease, the serum acid phosphatase never rose above 7.6 units, yet at autopsy there were widespread metastases to practically all tissues. The liver and parts of the bones of the lumbar spine, ribs and sternum were replaced to a considerable degree by large white masses of tumor tissue.

The latter case illustrates the dissociation between the extent of metastases and the level of serum acid phosphatase activity. Such a dissociation may be explained by either of two possibilities. First, the acid phosphatase does not pass readily from the metastatic tissue to the circulation. Second, metastatic tissue is an active producer of the enzyme, but the rate of excretion, metabolic degradation, or other mode of disposition of the enzyme may vary greatly with the patient and be excessive in some.

With regard to the first possibility, it is of interest that Nesbit *et al.* (N2) described a case of prostatic cancer in which extensive metastatic growth recurred in spite of palliative treatment with estrogen. The serum acid phosphatase level remained very low, but histochemical studies revealed an abundance of acid phosphatase in the epithelial cells of the primary and secondary tumors. According to Hertz *et al.* (H9), autologously transplanted prostatic tissue in dogs became well vascularized, secreted actively, and contained large amounts of acid phosphatase. However, the serum acid phosphatase remained low, even under the stimulus of androgen.

Some data are available concerning the metabolic degradation or other type of disposition of acid phosphatase in the circulation. London *et al.* (L12) studied in some detail the factors involved in the inactivation of serum acid phosphatase. *In vitro* experiments showed that the stability of the enzyme was dependent on the temperature, pH, and salt concentration. The rate of inactivation or denaturation at 37°C was monomolecular; and denaturation rate constants could therefore be derived for serums from normal persons or from various patients with benign hypertrophy or carcinoma of the prostate.

Of the several factors found to affect acid phosphatase *in vitro*, the one that could be tested safely *in vivo* was alteration of body temperature by application of the procedures of Ripstein *et al.* (R5). In two patients

with carcinoma of the prostate, lowering the body temperature caused a rise in serum acid phosphatase, and, conversely, raising the temperature led to a decrease. In one patient with a benign tumor of the prostate and in two normal individuals, all of whom had normal levels of serum acid phosphatase, lowering the body temperature had no effect on these levels. In another patient the spontaneous development of fever caused a marked decrease from 585 to 288 Bodansky units. Abatement of the fever was accompanied by a rise in the serum acid phosphatase to 566 Bodansky units (L11). In 1941, Huggins and his associates (H19) had made similar observations on the occurrence of fever in patients with prostatic cancer.

On the basis of clinical-biochemical correlations, Hudson *et al.* (H16) suggested that the liver may play a role in the metabolism of serum acid phosphatase of prostatic origin. A patient who was diagnosed clinically as having prostatic carcinoma was treated by enucleation prostatectomy, bilateral orchiectomy, and the institution of daily oral diethylstilbestrol. The surgical specimen showed a well differentiated adenocarcinoma of the prostate, but the serum acid phosphatase level was normal and there was no radiographic evidence of metastatic disease. Readmitted five and half years later because of acute urinary retention, there was still no radiological evidence of osteoblastic metastases, and the serum acid phosphatase was still low, 4.2 K.A. units. Several months later the patient was readmitted with a primary complaint of a sudden appearance and progressive enlargement of an epigastric mass. The liver was found to be enlarged, and several liver function tests had now become abnormal. The acid phosphatase had now risen to 201 K.A. units, and before death was 319 K.A. units. Autopsy revealed tumor infiltration to liver, lung, bladder, and lymph glands. The liver architecture was completely destroyed. A second case also showed parallelism between the rise in serum acid phosphatase and an increase in hepatic damage; whereas a third case demonstrated a parallelism between the fall of acid phosphatase and a return of liver function to normal.

6.6. Acid Phosphatase Activity in Nonprostatic Disease

6.6.1. *Introduction*

The term "serum" has been omitted designedly from the title of this section, for we shall be discussing not only alterations of acid phosphatase activity in the serum of patients with nonprostatic disease, but also in the leukocytes of patients with hematologic and hematopoietic disorders and, in some conditions, in certain specialized tissues.

6.6.2. *Serum Acid Phosphatase Activity in Skeletal Disease*

That occasional rises of serum acid phosphatase activity may occur in nonprostatic skeletal disease which may be associated with high serum alkaline phosphatase activity was first observed by Gutman and Gutman in 1938 (G11) and has since been confirmed by others (J1, S30). In a study of 32 cases of Paget's disease, Gutman *et al.* (G12) found that six of the most advanced cases with diseases of the skeleton and serum alkaline phosphatase levels over 70 Bodansky units had serum acid phosphatase levels above 3.0 K.A. units, the upper limit of normal. The highest activity recorded was 6.5 units. This compared with an incidence of five patients with elevated serum acid phosphatase activity in more than 200 control patients with diseases other than prostatic carcinoma or Paget's disease.

The possibilities that cryptic prostatic carcinoma may coexist with other diseases or that a high serum alkaline phosphatase activity possesses some residual activity at pH 5.0 tend to be negated by an analysis of these five patients. One female with carcinoma of the breast and osteolytic metastases of the femur, pelvis, and spine had an elevated serum acid phosphatase activity of 4.2 K.A. units, and a second female with an unknown primary but with osteolytic lesions of the ribs and scapula had an activity of 4.1 K.A. units. The serum alkaline phosphatase activities were 17.8 Bodansky units in the first case and 5.2 Bodansky units in the second case, both above 4.2 Bodansky units, the upper limit of normal values by this method.

One female and one male patient had hyperparathyroidism with elevated serum alkaline phosphatase activities and extensive bone changes characteristic of generalized osteitis fibrosa cystica. In both instances, the serum acid phosphatase activity of the serum fell to normal values after removal of the parathyroid adenoma despite transitorily increased serum alkaline phosphatase activity. The fifth patient was a female with osteopetrosis involving the major part of the skeleton. The serum acid phosphatase was 8.7 K.A. units, the highest in the control series—yet the serum alkaline phosphatase was within normal limits. It would appear, therefore, that some patients with skeletal disease may have a slight but definitely elevated serum acid phosphatase activity, at least as determined by the Gutman method (G10, G14), which cannot be explained by concurrent prostatic carcinoma or by a spillover of alkaline phosphatase activity to a pH of 5.0.

Table 10 shows the distribution of serum acid phosphatase activities in neoplastic disease other than prostatic cancer. The incidences of elevations were: 19% in patients with skeletal metastases; 2% in pa-

TABLE 10
PERCENTILE DISTRIBUTION OF SERUM ACID PHOSPHATASE IN NORMAL SUBJECTS AND IN PATIENTS WITH DISEASES OTHER THAN OF THE PROSTATE GLAND[a]

Condition	Number of patients	Percentage of cases with serum acid phosphatase		
		Less than 3.0 K.A. units	3.0–4.9 K.A. units	5.0–9.9 K.A. units
1. Normal subjects	30	100	—	—
2. Neoplasia other than prostatic carcinoma	240			
a. With skeletal metastases	99	81	13	6
b. With liver involvement	46	98	2	—
c. No bone or liver involvement	64	94	5	1
d. Primary bone tumors	31	90	10	—
3. Nonneoplastic disease of bone				
a. Paget's disease	96	79	18	3
b. Hyperparathyroidism	9	67	11	22
c. Miscellaneous diseases of bone	72	96	3	1

[a] Based on data of Sullivan *et al.* (S30).

tients with liver involvement; 6% in those without either bone or liver involvement; 10% in patients with primary bone tumors. In the category of nonneoplastic diseases of the bone, elevations were present in 21% of 96 patients with Paget's disease, in 33% of 9 patients with hyperparathyroidism, and in 4% of patients with miscellaneous diseases of the bone.

Using the Bodansky (B18, J2) procedure with β-glycerophosphate as substrate, Woodard (W8) was unable to obtain such elevations. She determined the serum acid phosphatase activities in 83 females and 342 males, or a total of 425 patients with miscellaneous diseases. Of these, 61 had various types of infectious or metabolic disorders, including 11 cases of inflammatory disease of bone and 12 cases of hepatic cirrhosis. The remainder had some type of neoplastic disease and about one-third had metastases to bone from cancer of various primary sites. There were 15 cases of osteogenic sarcoma and 32 cases of osteitis deformans. All these cases, whether their serum alkaline phosphatase activities were elevated or not, had serum acid phosphatase values that were essentially within the normal range, 0.06–0.89 Bodansky unit for females and 0.11–0.88 unit for males. In contrast to the Gutman method (G10, G14), there-

fore, the Bodansky method failed to show any elevations, however slight and infrequent, in nonprostatic neoplastic and other skeletal disease.

6.6.3. *Copper-Resistant Serum Acid Phosphatase in Miscellaneous Diseases*

Abul-Fadl and King (A4) had reported that a final concentration of 0.0002 *M* Cu^{2+} in the reaction mixture inhibited the hydrolysis of phenyl phosphate by human erythrocytic acid phosphatase to the extent of 85–95%, but exerted only a slight inhibition, approximately 8%, on the action by prostatic tissue. Aqueous extracts of liver, kidney, and various tumors were inhibited to the extent of 20–30%. Utilizing these findings and employing the Gutman method (G10, G14), Reynolds *et al.* (R4) studied the copper-resistant acid phosphatase levels in various miscellaneous diseases. The use of cupric ion was designed to minimize or even eliminate any serum acid phosphatase activity that had originated from red cells. The units of activity were expressed as the number of micromoles of phenol liberated from phenyl phosphate in 1 hour by 100 ml of serum. The serum acid phosphatase activity in 65 healthy volunteers was 11.3 ± 5.5 units. The upper limit of normal was 22.3 units. In a group of 104 female patients with acute or chronic inflammations, arthritis, osteoporosis, hypertension, and/or arteriosclerotic disease, diabetes mellitus, endocrine disorders, hepatic insufficiency or cirrhosis, renal disease, the activities ranged from 0 to 44.0 units, and 14 or 14% had elevated serum acid phosphatase activities, that is, values above 22.3 units. Twenty-one of 105, or 20%, of male patients with these miscellaneous conditions, had elevated serum acid phosphatase levels. This group included 13 patients with prostatic hypertrophy of whom 7, or about 50%, showed elevated activities.

Of considerable interest were the much higher incidences of elevated acid phosphatase activities in patients with nonprostatic, metastatic neoplastic disease. For example, in 70 female patients with metastatic carcinoma of the breast, the range of activities was 7.3 to 101 units, with 74% of the patients showing elevated activities. In 48 males with nonprostatic metastatic carcinoma and in 42 females with nonmammary metastatic carcinoma, the incidence of elevated acid phosphatase activities were 46% and 31%, respectively.

The report of these high incidences of elevations of acid phosphatase activity in miscellaneous disease and in nonprostatic neoplastic disease, determined by a method presumably more specific than the usual Gutman method (G10, G14), is not in accord with earlier studies, such as those of Sullivan *et al.* (S30) shown in Table 10. The incidences of elevations were considerably lower than those reported by Reynolds *et al.*

(R4). It might have been expected that, with the presumably more specific method proposed by these investigators, the reverse situation would have held. There appear to have been no further clinical studies utilizing the copper-resistant acid phosphatase method.

6.7. Serum and Plasma Acid Phosphatase Activity in Hematologic and Hematopoietic Disease

6.7.1. *Thrombocytopenia*

The development of platelets or thrombocytes takes place chiefly in the bone marrow from primitive totipotential reticulum or stem cells. The normal concentration of thrombocytes in the peripheral circulation of the adult is approximately 250,000–355,000/mm^3. Thrombocyte activity is necessary in the process of coagulation. Quantitative platelet deficiency or thrombocytopenia is one of the most common causes of hemorrhagic diathesis, and it may be due either to decreased platelet production or to increased platelet destruction.

Any of several basic disorders may account for decreased production. These may include congenital disorders such as hypoplastic anemia, hypoplastic thrombocytopenia, acquired conditions such as nutritional deficiency, toxic depression of the bone marrow, or the replacement of the bone marrow as in leukemia, carcinoma, granuloma, or fibrosis. Increased platelet destruction may include congenital disorders or such acquired disorders as chronic infections, portal hypertension, lymphomas, or thrombotic thrombocytopenia.

The presence of acid phosphatase in human platelets was first reported by Alexander (A6) in 1953. Shortly thereafter other investigators confirmed this finding (P1, S1). Using β-glycerophosphate as substrate, Zucker and Borelli (Z1) determined the acid phosphatase activity directly in platelets separated from human blood; these were washed three times with saline, frozen and suspended in saline in a concentration between 0.135 and 4.2 $\times$ 10^6 platelets per cubic millimeter. The activity, presumably calculated for 100 ml of a suspension containing 0.43 $\times$ 10^6 platelets per cubic millimeter, was 0.15 to 0.78 Bodansky units, as compared with a normal serum acid phosphatase activity of 0.0–0.8 Bodansky units. Pedrazzini and Salvidio (P1) also determined the acid phosphatase activity directly in platelets, expressing it as micrograms of P liberated in 1 hour from sodium β-glycerophosphate at pH 5.0 by 10^{10} platelets. The average value from 25 clinically normal individuals was 282 μg of P per hour.

In a subsequent study, Zucker and Woodard (Z2) prepared what they termed "platelet-poor plasma" by collecting the blood sample in silicone-

treated glassware, chilling half in ice water, and centrifuging at 4°C and 20,000*g* for 10 minutes. This sample was substantially free of platelets and other formed elements, and hence of acid phosphatase from these elements. On removal to room temperature, it underwent some clotting, and the resulting supernatant was termed serum from "platelet-poor plasma."

The remaining half of the sample collected in the silicone-treated glassware was transferred to ordinary glass and allowed to clot at room temperature for 30 minutes, as is usually done to obtain serum for acid phosphatase determinations. The mean values and their standard deviations for serums from "platelet-poor plasma" from various groups of subjects were as follows in Bodansky units: 28 normal women, 0.094 ± 0.009; 23 normal men, 0.109 ± 0.021. The corresponding values for ordinary serum acid phosphatase were 0.226 ± 0.13 and 0.278 ± 0.27. It appeared, therefore, that approximately 60% of the acid phosphatase in serum arises from the liberation of this enzyme from platelets as a result of clotting.

The moiety of serum acid phosphatase activity that may be derived from platelets has also been evaluated by Oski *et al.* (O2), employing the Gutman method (G10, G14). These investigators avoided the use of the vacuum tube (Vacutainer) and withdrew blood by syringe and gently ejected it into a glass tube containing ammonium oxalate–potassium oxalate as anticoagulant. The blood was immediately centrifuged at 4°C for 15 minutes at 700*g*; the supernatant plasma was removed without disturbing the buffy coat, transferred to a plastic tube, and recentrifuged at 4°C for 30 minutes at 4000*g*. The plasma was aspirated and found to be free of platelets both by direct platelet counting and by examination of a Wright-stained smear of a centrifuged aliquot.

The mean values and the standard deviations for this platelet-free plasma acid phosphatase were determined for groups of children at various ages and for adults. Each group consisted of 10 or 11 individuals. The values, expressed as K.A. units, were as follows: 1.0–3.0 year olds, 4.5 ± 1.0; 3.1–6.0 year olds, 4.2 ± 0.7; 6.1–9.0 year olds, 4.1 ± 0.8; 9.1–16.0 year olds, 3.9 ± 0.9 K.A. units. The value for male adults were 2.3 ± 0.3 K.A. units and that for female adults, 2.2 ± 0.3. The mean plasma acid phosphatase activities declined with increasing age. Although the activity of plasma acid phosphatase was less than the corresponding serum activity, these differences were not large, ranging from 0.1 to 1.9 units.

Reports on the alteration of serum and/or plasma acid phosphatase activity in thrombocytopenia have not always been consistent. Zucker and Woodard (Z2) described a series of 12 patients with thrombocyto-

penia secondary to a variety of conditions such as carcinoma of the breast, acute and chronic leukemias with platelet counts chiefly between 2000 and 60,000. The mean value for the acid phosphatase activity in serum from platelet-poor plasma was essentially normal, but the value for the activity in ordinary serum was 0.123 Bodansky unit, significantly less than the values of 0.226 unit for normal women and 0.278 unit for normal men. These findings indicated again that the platelets were a major source of the acid phosphatase liberated into serum during clotting (Z2).

Oski *et al.* (O2) classified their cases of thrombocytopenia as arising from impaired production of platelets, from increased destruction of platelets, or from a combination of these factors. In a group of 8 children with bone marrow failure, evidenced by low platelet counts and megakaryocytic hypoplasia or aplasia without complicating infection or drug therapy, each patient had a plasma acid phosphatase activity lower than the mean value for its age. The differences were, however, not large. For example, in a child with acute leukemia aged 2 years and 7 months, the platelet count was 30,000 and the plasma acid phosphatase was 3.6 K.A. units, as compared with a value of 4.5 ± 1.0 K.A. units for normal children in this age group. The comparison of the plasma acid phosphatases in this group of 8 children, as a whole, with those in the normal group yielded a significant p value of less than 0.01. In general, therefore, these findings are in agreement with those of Zucker and Woodard (Z2) for patients with thrombocytopenia due to bone marrow failure.

In a group of six children with acute thrombocytopenia and bone marrow megakaryocytic hyperplasia, the blood plasma, prepared as previously described, showed, in each case, an acid phosphatase activity, as determined by Gutman's method, that was higher than the mean value for that age ($p = 0.02$). The p value for the comparison of the group as a whole with normals was between 0.01 and 0.02. In all six of these patients the plasma acid phosphatase values returned to normal or near normal levels as the thrombocytopenia was corrected. Oski *et al.* (O2) also studied 15 cases of chronic idiopathic thrombocytopenic purpura in whom the bone marrow showed normal to increased numbers of megakaryocytes. Of these, 13 showed plasma acid phosphatase values that were elevated above the normal mean for their age, albeit some of these differences were small. However, these elevations were statistically significant with a p value less than 0.01.

In essence, then, these investigators felt that the plasma acid phosphatase activity, unobscured by *in vitro* destruction of platelets, could reflect the contribution of acid phosphatase from *in vivo* platelet destruction in various types of thrombocytopenias. To summarize, in thrombocyto-

penias with a deficiency of megakaryocytes in the bone marrow, the plasma acid phosphatase activity was significantly lower than that in normal controls, whereas it was higher than normal in patients with chronic or idiopathic thrombocytopenic purpuras and normal or increased numbers of megakaryocytes in their bone marrow.

However, Cooley and Cohen (C9) studied nine cases of idiopathic thrombocytopenic purpura in which they failed to find any consistent correlation between the plasma acid phosphatase activities and platelet counts. Cohen *et al.* (C6) had shown that this condition could be classified into two major types, destructive and nondestructive, comprising 80% and 20%, respectively, of the total. In sequential studies of their various patients, Cooley and Cohen (C9) did not find any increased plasma acid phosphatase activity in those showing the destructive type, although in two cases of nondestructive (hypoplastic) thrombocytopenias, the plasma acid phosphatase activities were usually normal or low. Cooley and Cohen (C9) also found that in a group of eight patients with secondary (nondestructive) thrombocytopenias with nearly normal platelet life-spans (mostly 5–7 days) the plasma acid phosphatase levels tended to be low and to be correlated with the platelet count.

Pedrazzini and Salvidio (P1) found that the average value of acid phosphatase activity in platelets from 25 patients with hypoprothrombinemia secondary to Morgan-Laennec's hepatic cirrhosis was 119 μg of P per hour per 10^{10} platelets, significantly less than the normal value of 282 μg of P. These investigators also found that ATPase, 5′-nucleotidase, and alkaline phosphatase activities were reduced in the platelets of the patients with liver cirrhosis by 44%, 58%, and 67%, respectively. Along with the reduction in acid phosphatase, these changes were considered to be an expression of functional damage to the platelets.

6.7.2. *Serum and Plasma Acid Phosphatase in Thrombocytosis*

The presence of greater than normal amounts of thrombocytes in the circulation is known as thrombocytosis and along with reticulocytosis and leukocytosis is a manifestation of increased activity of the hematopoietic system. Zucker and Woodard (Z2) reported a series of 12 patients with thrombocytosis, consisting of two cases of polycythemia vera, three of essential thrombocytemia, three of chronic granulocytic leukemia, one myeloproliferative syndrome, one erythroleukemia, and one cancer of the bladder. The platelet counts ranged from 685×10^3 to 2500×10^3 per cubic millimeter, all much above the upper limit of normal.

The serum acid phosphatase activity was determined by the Bodansky method (B18, J2) with β-glycerophosphate as substrate. The mean value for the series of 12 patients was 0.983 ± 0.122 Bodansky unit, consider-

ably elevated above the normal mean values of 0.226 ± 0.0126 unit for women and of 0.278 ± 0.0270 unit for men. This elevation represented the release on clotting of acid phosphatase from the large number of platelets. The mean value for acid phosphatase activity of platelet-poor plasma in the series of these 12 patients with thrombocytosis was 0.141 ± 0.0162 unit and was not significantly different from the mean values of 0.094 ± 0.0091 unit for normal women, 0.109 ± 0.021 unit for normal males or of 0.134 ± 0.018 unit for patients with thrombocytopenia. Accordingly, it may be concluded that in thrombocytosis there is little *in vivo* contribution by the platelets to the circulating acid phosphatase.

6.7.3. *Serum Acid Phosphatase Activity in Other Myeloproliferative Disease*

The term "myeloproliferative disorders" has been applied to all those conditions which are characterized by proliferation of cells in bone marrow or in other sites of extramedullary blood formation. The overgrowth is self-perpetuating and involves one or more lines of bone marrow elements (myelocytic, erythrocytic, megakaryocytic) and cells like fibroblasts derived from the reticulum. We have already mentioned some of these conditions, for example, chronic granulocytic leukemia and myeloid metaplasia, in connection with our discussion on the relationship between serum acid phosphatase and the platelet count.

Employing β-glycerophosphate as substrate in the Bodansky method, Bases (B6) found that 9 of 16 patients had slight, but significant, elevations of serum acid phosphatase activity—in one case as high as 4.7 Bodansky units. In general, a proportionality existed between the enzyme activity and the platelet or white blood cell count. In sequential studies of individual patients, particularly in connection with a chemotherapeutic regimen, the change in phosphatase activity paralleled both the white cell and platelet counts, but occasionally the parallelism existed only between the enzyme activity and the white cell count. In this connection it may be recalled that, according to Zucker and Borelli (Z1), the clotting of blood involves the destruction of platelets and possible release of their acid phosphatase. Valentine and Beck (V1) demonstrated that white cells from patients with chronic granulocytic leukemia in general had abnormally high levels of acid phosphatase. It would appear, therefore, that in Bases' cases of myeloproliferative disease, both the platelets and the granulocytes might have been the source of the acid phosphatase elevations (B6).

Bases (B6) found no elevations of the serum acid phosphatase activity in a group of 20 patients with chronic lymphocytic leukemia, other lymphomas, leukemoid reactions, and acute leukemia. This would ap-

pear reasonable in view of the findings that leukocytes from patients with acute leukemia or lymphatic leukemia either had normal or low acid phosphatase activity, as determined with β-glycerophosphate as substrate (B10, V1). However, Klastersky and Coune (K8) recently reported a case of a male with lymphoblastic leukemia and elevated values of serum acid phosphatase, ranging from about 4 to 8 units; nitrophenyl phosphate was used as the substrate. These elevated values decreased and even reverted to normal values, 0.0 to 0.63 unit, after administration of vincristine. The decrease in enzyme activity paralleled the decrease in the number of blasts in the peripheral blood and percentage in the marrow. During continued treatment for 4 months, the number of blasts, their percentage in the marrow, and the serum acid phosphatase activity continued at low levels. At the end of this period, the patient became resistant to vincristine, and the blasts and the serum acid phosphatase activity rose. Again, the values for these parameters fell with the institution of methotrexate therapy, but death occurred following massive hematuria. Postmortem examination showed leukemic infiltration of the spleen, bone marrow and laterotracheal nodes, but the prostate was clear of carcinoma or of leukemic filtrate. Although the serum acid phosphatase activity was inhibited by L-(+)-tartrate, and spleen acid phosphatase has also been reported to be inhibited by this compound (A4), it is difficult to ascribe any definite basis for the increased serum acid phosphatase in the case reported by Klastersky and Coune (K8).

6.8. Acid Phosphatase Activity in Gaucher's Disease

6.8.1. *Serum Acid Phosphatase in Gaucher's Disease*

The characteristic lesion in this disease is the infiltration of spleen and, to a lesser extent, of liver, bone marrow, and lymph nodes with Gaucher cells. These cells have small dark central or eccentric nuclei and clear or foamy cytoplasm with fibrillar striations. Gaucher's disease is one of the lipidoses and is caused by an inherited deficiency of glucocerebrosidase, the enzyme required for the degradation of glucocerebroside (B28). As a result of the phagocytosis of cells and cellular debris, large amounts of glucocerebroside are deposited in the reticuloendothelial cells, causing them to assume the typical wavy, fibrillar appearance of Gaucher's cells.

In 1957, Tuchman and Swick (T6) reported the finding of elevated serum acid phosphatase activities, ranging from 7.0 to 10.3 K.A. units in a 68-year-old man, who was first suspected of having carcinoma of the prostate. The diagnosis of Gaucher's disease was then considered and confirmed by a sternal marrow aspiration. By 1959, Tuchman *et al.* (T7)

had collected a series of 12 patients who showed a range of 7 to 14.3 K.A. units of serum phosphatase activity when determined by the method of Gutman (G10, G14). Tuchman *et al.* (T7, T8) stated that the upper limit of normal in their hospital laboratory was 4 K.A. units and that a range between 4 to 5 K.A. units was equivocal. Neither Cu^{2+} or tartrate reduced any, and formaldehyde reduced only one, of the 12 activities to below 5 K.A. units, thus characterizing this acid phosphatase activity as different from the acid phosphatases of erythrocyte liver, prostate, spleen, or bone marrow.

Using β-glycerophosphate as substrate, Tuchman *et al.* (T7) reassayed the serum acid phosphatase activities in his series of 12 patients with Gaucher's disease. In contrast to the Gutman method, which had yielded elevated values in all cases, only four of the patients now showed elevations above the upper limits of normal, approximately 0.8 Bodansky unit. The two patients who had shown the highest values, 10.2 and 14.3 K.A. units by the Gutman method (G10, G14) had one normal value, 0.75 Bodansky unit, and one slightly elevated value, 0.88 unit. Nonetheless, elevation of serum acid phosphatase activity in Gaucher's disease with phenyl phosphate as substrate cannot be considered as nonspecific or spurious. Tyson *et al.* (T9) reported the case of a 65-year-old man who was considered to have cirrhosis of the liver because of hepatosplenomegaly, anemia, pancytopenia, and esophageal varices. On admission to the hospital three years later, skeletal survey revealed some areas of lucency in the femurs compatible with Gaucher's disease, multiple myeloma, or myeloproliferative diseases. The serum acid phosphatase level was consistently elevated, from 12.9 to 13.7 K.A. units and about 2.0 Bodansky units. Sternal marrow biopsy was refused by the patient. After his death five years later, autopsy and microscopic examination revealed infiltration of the liver, spleen, bone marrow, and lymph nodes with typical Gaucher cells.

It has already been noted that Gaucher's disease is characterized by infiltration of the spleen and, to a lesser extent, of the liver, bone marrow, and lymph nodes with the typical Gaucher cells. Confirming earlier observations that these cells stained for acid phosphatase, Crocker and Landing (C10) suggested the possibility that the elevated serum acid phosphatase activity in patients with Gaucher's disease might reflect a spillage of the enzyme from the spleen and other tissues. Using β-glycerophosphate as a substrate, these investigators obtained average activities of 330 Bodansky units per gram for spleens from 4 children with miscellaneous disease, of 279 units per gram from 4 children with Niemann-Pick's disease, and a markedly elevated value of 875 units per gram from spleens from 7 children with Gaucher's

disease. Five of the children in this last group, all of whose serum acid phosphatase activities were elevated, as determined by the Gutman method with phenyl phosphate as substrate (G10, G14), were subjected to splenectomy. In four of these, the serum acid phosphatase activity decreased precipitously by 40–55%.

6.8.2. *Isoenzymes of Acid Phosphatase in Serum and Spleen Cell Suspensions of Gaucher's Disease*

In 1964, Czitober *et al.* (C11) reported three patients with Gaucher's disease in whom the serum acid phosphatase exhibited five zones of activity. Using disc electrophoresis on polyacrylamide-gel columns, as described by Davis (D5), Goldberg and his associates (G6) determined the isoenzyme pattern in the serums of nine patients with Gaucher's disease who had elevated serum acid phosphatase activities ranging from 8 to 25.8 K.A. units. In the group as a whole, five bands of acid phosphatase activity were discernible. The most anionic migrated in a position similar to transferrin and was designated as band I. Band II was in the fast moving haptoglobulin region, and bands III, IV, and V had mobilities of γ-globulins and the slower haptoglobulins. All nine patients had at least two bands, I and II, of which the latter was the broadest and most intensely stained. One or more of the minor bands, III–V, were considerably narrower and were present in five of the nine patients. Of these five patients, three had three bands and two patients had two minor bands. One patient showed all five bands.

Li *et al.* (L8) studied spleen cell suspensions, with and without cotton filtration, from a patient with Gaucher's disease. The preparation, without filtration, showed a strong isoenzyme 1 and a new isoenzyme, which remained at the cathode. This isoenzyme was distinctive, not being found in leukocytes, and was designated as isoenzyme No. 0. It should be noted that Li *et al.* (L7, L8) designated the isoenzymes as ranging from No. 1, cathodic, to No. 5, the most anionic. After removal of the Gaucher cells from the spleen cell preparation by cotton filtration, the remaining leukocytes did not show isoenzyme No. 0, but isoenzymes Nos. 1, 2, 3, and 4 were now apparent. Isoenzyme No. 0 was therefore characteristic of the Gaucher cell.

6.9. Leukocytic Acid Phosphatase Activity in Hematologic and Hematopoietic Disease

6.9.1. *Leukocytic Acid Phosphatase in Normals*

The acid phosphatase activity of leukocytes was determined by Valentine and Beck (V1) in 1951 and expressed as the amount of phos-

phorus liberated from 0.02 *M* sodium β-glycerophosphate in 1 hour at 37°C by 10^{10} cells. The reaction mixture was buffered with acetate-Veronal at pH 5.0 and contained 1 mg per milliliter of a solution of saponin to lyse the cells. The range in a series of 23 controls was 14–37 mg, and the mean was 22 mg. Using the method of Allen and Gockerman (A7), Li *et al.* (L7) recently found the average for the total acid phosphatase activity of the leukocytes from five normal persons to be 1219 nmoles of naphthol liberated from 0.005 *M* sodium α-naphthyl acid phosphate per hour per 10^7 cells at pH 5.0 and 25°C.

Electrophoresis was carried out by Li *et al.* (L7) by the method of Axline (A18). Optimal separation was obtained at pH 4.0 on a 7.5% acrylamide gel matrix containing 0.5% Triton X-100. Electrophoresis was carried out at 4°C for 50 minutes with a current of 4 mA/tube. Four isoenzymes of acid phosphatase were obtained for normal leukocytes, proceeding from the cathodic, No. 1, to the most anionic, No. 4. The mean normal values for the distribution of activity among these isoenzymes were No. 1, 37.8%; No. 2, 29.2%; No. 3, 11.5%; No. 4, 21.5%. In a subsequent study, Li and his associates (L8) found that preparations of 10×10^6 lymphocytes or of 10×10^7 platelets per milliliter of 5% Triton X-100 each gave, upon electrophoresis, only one band of isoenzyme activity, No. 3. It was the neutrophiles and monocytes that contributed isoenzymes 1, 2, and 4. In normal cases the neutrophiles were as high as 70–80%. The acid phosphatase activities of these bands in neutrophiles or monocytes were sufficiently high so that even as little as 5% of either cell type in the blood of patients with chronic lymphocytic leukemia yielded clear evidence of isoenzymes 1, 2, and 4.

6.9.2. *Leukocytic Acid Phosphatase in Leukemia*

In 1951, Valentine and Beck (B8, V1) found that, in spite of considerable interindividual variability, leukocytic acid phosphatase was elevated in chronic granulocytic leukemia and tended to be decreased in chronic lymphocytic leukemia and in acute leukemia (Table 11). Statistical treatment of their original data shows that only the mean values for alterations in chronic granulocytic (myelocytic) leukemia and acute leukemia were significantly different ($p < 0.01$) from the mean value for leukocytic acid phosphatase in normals. Using the method of Allen and Gockerman (A7) and α-naphthyl phosphate as substrate, Li *et al.* (L7) found, in agreement with Valentine and Beck (V1), that the total leukocytic acid phosphatase activity was decreased in acute granulocytic and lymphocytic leukemias. However, in contrast to the findings of Valentine and Beck (V1), Li *et al.* (L7) observed no elevation in

TABLE 11
ACID PHOSPHATASE IN THE LEUKOCYTES OF NORMAL SUBJECTS AND PATIENTS WITH LEUKEMIA[a]

Group	No. of subjects	No. of determinations	Range	Mean
Normals	23	23	14–37	22
Leukocytosis	30	30	7–66	26
Chronic granulocytic leukemia	14	22	16–61	35
Chronic lymphocytic leukemia	12	16	1–106	18
Acute leukemia	8	14	0–46	9

[a] Data of Valentine and Beck (VI) and of Beck and Valentine (B8). Activities are expressed as milligrams of phosphorus liberated in 1 hour by 10^{10} cells from a reaction mixture at pH 5.0, containing a final concentration of 0.02 *M* sodium β-glycerophosphate as substrate and 1 mg per milliliter of saponin to lyse the leukocytes.

the mean value for chronic granulocytic leukemia (Table 12). It was also of interest that the total activity was greatly reduced in chronic lymphocytic leukemia. The distribution of the four normal isoenzymes in various types of leukemia is also shown. Electrophoresis was carried out on polyacrylamide gels by the method of Barka (B3); the substrate was sodium α-naphthyl acid phosphate, and the diazonium salt of *O*-aminoazotoluene (fast garnet GBC) was the coupler. The relative intensity of each phosphatase band in the gels was estimated with a Gilford densitometer. The shift in isoenzyme pattern from the normal may be readily appreciated (Table 12). Thus, in acute granulocytic and chronic lymphocytic leukemias, the fraction of isoenzyme 3 was increased, whereas that of isoenzyme 2 was decreased substantially.

In addition to the four bands of acid phosphatase isoenzyme activity in normal leukocytes, two other isoenzymes may appear in leukemia. In a case of acute granulocytic leukemia with 100% blast forms, only one electrophoretic band of activity was manifest; this migrated between normal band 3 and 4 and was therefore designated 3b. In a case of leukemic reticuloendotheliosis with 98% reticulum cells, only one, and this a strongly staining band, was present; this migrated anodically beyond No. 4 and was therefore designated No. 5 (L8).

6.9.3. *Leukocytic Acid Phosphatase in Leukemic Reticuloendotheliosis*

This condition—which has also been known under the names of "reticulum cell leukemia," "lymphoid reticular cell neoplasia," and "hairy cell disease" (M8)—is characterized by massive splenomegaly

TABLE 12

LEUKOCYTE ACID PHOSPHATASE ACTIVITY IN VARIOUS HEMATOLOGIC DISEASES[a]

Disease	No. of cases	Total activity[b]	Fractions				
			1 (%)	2 (%)	3 (%)	4 (%)	5 (%)
Normal	5	1219 (926–1650)[d]	37.8 (28–48.8)	29.2 (25–36.7)	11.5 (8.2–15.7)	21.5 (17.3–26)	Trace[c]
Chronic granulocytic leukemia	4	1242 (826–1700)	35.1 (23.7–48.3)	32.6 (27.5–38.6)	7.3 (5.0–9.7)	25 (17.5–32.7)	—
Acute granulocytic leukemia	3	649 (120–1300)	35.2 (17.5–53.1)	12.7 (10.9–14.5)	29.4 (15.6–47.4)	22.7 (18.8–25.1)	—
Acute lymphocytic leukemia	1	826	29.2	39.7	8.0	23.1	—
Chronic lymphocytic leukemia	7	284 (33–836)	29.9 (0–49.7)	14.7 (0–29)	42.5 (13.6–100)	12.9 (0–23.9)	—

[a] Based on data of Li *et al.* (L7).

[b] Expressed as nanomoles of naphthol per hour per 10^7 cells.

[c] Detected in one case.

[d] Numbers within parentheses indicate range.

due to invasion of reticulum cells. The peripheral blood and bone marrow contain large numbers of a typical large "lymphoid reticular cell," 12–20 μ in diameter, with a round or oval, occasionally kidney-shaped, eccentrically placed nucleus and a plentiful, very faintly basophilic and cloudy cytoplasm (L5). Ten of a series of 25 patients, recently reported by Lee *et al.* (L5), had moderate anemia, and all but 3 had varying degrees of thrombocytopenia. At the first visit, 14 of the 25 patients had reduced total white blood cell counts, less than 5000 per cubic millimeter. In all patients, the differential white cell count was very abnormal.

As was noted above, a preparation obtained from patients' peripheral blood in which 98% of the leukocytes were reticulum cells, showed only one isoenzyme, No. 5, of acid phosphatase activity (L8). In patients with a differential white cell count with lesser numbers of reticulum cells and greater numbers of neutrophiles and lymphocytes, isoenzymes 1, 2, 3, and 4 were also evident. For example, in a case with 54% reticulum cells, 20% neutrophiles, 1% monocytes, and 25% lymphocytes, the relative isoenzyme activities were: No. 0, 0%; No. 1, 30.8%; No. 2, 18.8%; No. 3, 9.7%; No. 3B, 8.0%; No. 4, 10.8%; No. 5, 21.9% (L8).

L-(+)-Tartrate (0.05 *M*) inhibited isoenzymes Nos. 1–4 but had no appreciable effect on the reticular cell isoenzyme, No. 5 (M8, Y1). In cytochemical studies of blood smears from three patients with leukemic reticuloendotheliosis, the acid phosphatase activity in the monocytes, eosinophiles, neutrophiles, and other cells that could definitely be identified as lymphocytes did not differ appreciably from those of normal subjects. In all three patients the neoplastic reticulum cell showed various degrees of acid phosphatase activity; most of them were strongly positive. The enzyme activity in these cells was resistant to to L-(+)-tartrate, whereas it was completely inhibited in other types of cells.

6.9.4. *Leukocytic Acid Phosphatase in Other Hematologic Disease*

Li *et al.* (L7) have also studied the leukocytic acid phosphatase activity and the distribution of its isoenzymes in other hematologic diseases. The average values for the total activity and the distribution of the four isoenzymes in three cases of hemochromatosis were not significantly different from those observed in normals (Table 11). This also held for the averages and ranges for three cases of polycythemia vera. However, the average value, 5.7%, and the range, 4.5–7.3%, for isoenzyme 3 in three cases of Hodgkin's disease appeared lower than the corresponding values, 11.5% with a range of 8.2–15.7% for the five normal individuals (Table 12). Three cases of infectious mononucleosis had

an average activity of 721 (424–1000) nmoles of naphthol liberated per hour per 10^7 cells, which was distinctly lower than the normal value. The distribution of activity for the four isoenzymes was: No. 1, 51.4%; No. 2, 12.8%; No. 3, 19.5% and 16.3%, with isoenzyme 1 activity appearing distinctly higher, and isoenzyme 2 activity distinctly lower, than the corresponding values for normals (Table 12).

6.10. Serum Acid Phosphatase in Thromboembolism

Schoenfeld *et al.* (S7, S8, S9) have reported the occurrence of small but definite elevations of serum acid phosphatase activity, as determined by the Gutman method (G10, G14), in myocardial infarction, pulmonary embolism, peripheral thromboembolism and arterial embolism. The following possible explanations may be invoked for this phenomenon: (a) degeneration of enzyme-rich parenchymal tissue subserved by the occluded vessel; (b) autolysis of enzyme-rich cells, including platelets and erythrocytes, enmeshed in the blood clot; (c) generalized hypoxic injury to, and release of acid phosphatase from, various organs; (d) thrombocytosis caused by stress-induced splenic contraction and by increased thrombocytogenesis due to tissue necrosis. However, not sufficient evidence was available to decide among these alternatives, or to assess the contributions of each to the elevated serum acid phosphatase level.

6.11. Serum Acid Phosphatase in Diseases of Childhood

Although serum acid phosphatase activity has already been considered in certain diseases of childhood, such as leukemia or Gaucher's disease, it may be of value to make several general comments in this area. Laron and Kowadlo (L4) found that the mean normal values for total serum acid phosphatase were: 5.23 ± 1.26 K.A. units for children 1 year of age and 4.63 ± 0.93 K.A. units for children 2–8 years of age. These are higher than the values for adults. The mean value for L-(+)-tartrate-inhibited acid phosphatase activity was 0.21 ± 0.28 K.A. units for all age groups.

Of 24 children with rheumatic fever, 6, or 25%, had total serum acid phosphatase activities above 6.5 K.A. units or the mean normal value, 4.63 K.A. units plus 2 standard deviations. In 2 of 9 infants with rickets, the total serum acid phosphatase activity was abnormally high, 11.3 and 9.7 K.A. units. High values were also obtained in occasional cases of nephrosis, pneumonia, and hepatitis. Of 23 children with rheumatic fever in which tartrate-inhibited serum acid phosphatase activity was determined, 12 had values above 0.77 K.A. unit, or the mean value, 0.21 K.A. unit plus two standard deviations.

7. Lysosomal Disease and Acid Phosphatase Activity

7.1. Introduction

During the past several years a number of reports have appeared indicating that lysosomes and the acid hydrolases characteristic of them may play a role in several types of human disease. Weissmann (W3) has suggested that lysosomes may be an important factor in autoimmune phenomena and connective tissue diseases, such as systemic lupus erythematosus, rheumatic fever and polymyositis. According to this thesis, degradative enzymes, released from lysosomes, may denature the native constituents of cells or connective tissue. Such denatured products could then induce the formation of circulating antibodies, which would be directed not only against denatured constituents but also against antigenically related normal tissues. Gordis (G8) has further reviewed the pathological effects of lysosomal abnormalities, such as deficiency or excess of hydrolases, the stability of lysosomes, and the possible types of disease each of these effects would lead to.

7.2. Lysosomes and Cancer

The selective concentration of hydrocarbon carcinogens in lysosomes and the production of chromosomal damage following lysosomal disruption have been reported by Allison and his associates (A8, A9). Scherstén *et al.* (S5, S6) have shown that both the free and total activities of the lysosomal enzymes—acid phosphatase, aryl sulfatase, cathepsin, and β-glucuronidase—in the livers of patients with malignant renal tumors were significantly elevated. In kidney tissue without tumor growth, these lysosomal enzymes were of the same order of magnitude as in normal tissues. The enzyme activities in renal carcinoma tissue were lower than in liver and in kidney tissue. Scherstén *et al.* (S6) suggested that lysosomal enzymes may be released from tumor tissue and then taken up by other tissues.

7.3. Deficiency of Lysosomal Acid Phosphatase

In 1970, Nadler and Eagan (N1) described a new familial metabolic disorder which, quite accidentally, was found to be characterized by a deficiency of lysosomal acid phosphatase activity. This finding was observed in the male child resulting from the fourth pregnancy of a 34-year-old Puerto Rican woman. The previous three pregnancies had resulted in the birth of children who had survived for periods ranging from 2 hours to 11 months. The full-term male infant resulting from the fourth pregnancy behaved normally at first, but developed lethargy

and incidents of vomiting at about the age of 3 months and was admitted at this time because of these symptoms and fever. The liver was enlarged to 4 cm below the right costal margin. He died several days after admission.

In the course of studies of the nuclear, mitochondrial, lysosomal, microsomal, and supernatant fractions of the homogenates of fibroblasts grown from skin biopsies, it was found that the activities of glucose-6-phosphate dehydrogenase, lactic dehydrogenase, α-glucosidase, and β-glucuronidase were normal. In contrast, the activity of acid phosphatase was reduced in all fractions and was virtually absent in the lysosomal fraction. Addition of Triton did not appreciably increase the lysosomal acid phosphatase activity. Table 13 shows that the acid phosphatase activities in the lysosomal fraction from the two obligate heterozygotes (the parents, who were first cousins) and in five other members of the family (presumably heterozygotes) were approximately 50% of the activities in the control family. The mode of inheritance was therefore apparently that of an autosomal recessive defect.

The acid phosphatase activities were also determined in brain, kidney, liver, and spleen taken at autopsy from the patient and, like the acid phosphatase activities of lymphocytes and the fibroblasts, was found to be virtually absent, certainly less than about 2% of the activities in the corresponding tissues of the control individuals. Lymphocytes obtained from whole blood showed essentially the same acid phosphatase activity in the heterozygotes as in the controls. However, after 56 hours of stimulation with phytohemagglutinin (PHA), the acid

TABLE 13
ACID PHOSPHATASE ACTIVITY OF CULTIVATED FIBROBLASTS IN LYSOSOMAL ACID PHOSPHATASE DEFICIENCY[a]

Subjects	Original homogenate	Lysosomal fraction	Lysosomal fraction + Triton
Controls (15)[b]	6.2 ± 0.5[d]	7.2 ± 0.7	9.6 ± 1.9
Obligate heterozygotes (2)	2.8 ± 0.2	3.0 ± 0.9	4.2 ± 1.3
Presumable heterozygotes (5)	2.7 ± 0.3	3.3 ± 1.1	4.3 ± 1.5
Patient (1)	1.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Abortus (1)[c]	1.0 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

[a] Based on data of Nadler and Eagan (N1).
[b] Figures in parentheses indicate the number of patients studied.
[c] Detected *in utero* by examination of amniotic fluid cells at week 13 of pregnancy.
[d] Values are expressed as micromoles of *p*-nitrophenol formed per hour per microgram of protein.

phosphatase activity of the lymphocytes in the heterozygotes showed about one-third the activity of that in the controls.

The report by Nadler and Eagan (N1) raises certain general questions about the metabolic role of acid phosphatase. It is at present difficult to determine how intermittent vomiting, hypotomia, lethargy, opisthotonus, and terminal bleeding may be related to the absence of lysosomal acid phosphatase. Yet a few gleanings from the literature indicate that some general cellular metabolic role for acid phosphatase may exist. As was previously noted, DiPietro and Zengerle (D13) were able to separate and purify three isoenzymes of acid phosphatase from the human placenta. Isoenzyme III possessed properties of potential metabolic interest in that it was activated to a considerable extent by the purines, adenine, and 6-ethylmercaptopurine and was inhibited by pyridoxine 5-phosphate (vitamin B_6). However, these activities were not observed with organic phosphate substrates other than *p*-nitrophenyl phosphate. It has also been suggested that acid phosphatase may participate in the regulation of pyridoxal phosphate-requiring enzymes (A10).

7.4. Multiple Lysosomal Enzyme Deficiency

Within the past few years, several patients have been described who have a hereditary defect characterized by severe psychomotor retardation, shortness of stature, intermittent respiratory infections, and slowly progressive "Hurler-like" changes of facies and bony configuration. Fibroblasts grown from a skin biopsy have inclusion bodies and, hence, have been considered to display the "I (for inclusion) cell phenomenon." The disease has also been referred to as "I-cell disease."

A number of lysosomal enzymes in the fibroblasts of the patient have been found to have greatly reduced activities. Thus, in a recent study, Wiesmann *et al.* (W5) reported that arylsulfatase A activity was reduced to 20% of normal; β-galactosidase, *N*-acetyl-β-galactosaminidase, *N*-acetyl-β-glucosaminidase, and β-glucosaminidase activities were as low as 2–10% of normal, and α-fucosidase was not detectable. Intermediate enzyme activities were found in the fibroblasts of the mother of the patient. Nonlysosomal enzymes, such as malic dehydrogenase and lactic dehydrogenase, showed normal activity but curiously enough, the typically lysosomal enzyme, acid phosphatase, also showed normal activity.

Testing of the medium in which the fibroblasts had been growing for 3 days revealed significantly elevated levels of activity for the lysosomal enzymes that had been decreased within the cell. No mention was made of acid phosphatase activity. Wiesmann *et al.* (W5) considered several

possible explanations, but concluded the most likely one to be a basic defect in the passage of lysosomal enzymes into the outward medium. However, no explanation was offered for the absence of any change in the acid phosphatase activity. Sawant *et al.* (S3) have investigated several factors that affect the permeability of the rat liver lysosomal membrane and the rates of outward passage of the various lysosomal enzymes. It is possible that, if lysosomal permeability be a characteristic of "I-cell disease," such permeability is not general and does not apply to acid phosphatase. Again, Rahman *et al.* (R1) has reported that rat liver lysosomes may be heterogeneous in terms of their enzyme contents. If such a consideration were to apply to lysosomes from other tissues and species, it is conceivable that lysosomes containing β-galactosidase and the other enzymes which are reduced in activity would be damaged whereas those containing acid phosphatase would be more resistant.

7.5. Hemorrhagic Enteropathy and Lysosomal Enzymes

In the past few years, there has been an increasing interest in the hemorrhagic enteropathy that may be associated with myocardial infarction, terminal cardiac diseases which involve heart failure, valvular heart disease with or without operation, and open-heart surgery (C2). The damaged intestinal mucosa cells may release their potent hydrolytic lysosomes into the lumen and, indeed, into the blood through the thoracic duct (B13).

Chiu *et al.* (C2) have recently studied the intestinal lesions and circulating lysosomal enzymes in extracorporeal circulation both in experimental and in clinical situations. With respect to the latter, 25 adult patients undergoing cardiopulmonary bypass for open-heart surgery were investigated. Arterial blood samples were obtained prior to and immediately after cardiac bypass, and several hematologic and biochemical parameters, including serum acid phosphatase and β-glucuronidase, were determined. Serum acid phosphatase activity was determined by a commercially available method with *p*-nitrophenyl phosphate as substrate (C2) and β-glucuronidase by the method of Fishman *et al.* (F4).

There was no significant difference between the serum β-glucuronidase activities before and after the bypass. However, the serum acid phosphatase was higher, in each of the patients but one, after the bypass than before, and the mean value was significantly higher. The basis for the increased serum activity of one lysosomal enzyme, acid phosphatase, but not that of another, β-glucuronidase, is of interest. It is possible, as Chiu *et al.* (C2) have suggested, that the increased acid phosphatase

activity may reflect largely the acid phosphatase arising from hemolyzed red cells. On the other hand, if lysosomes are considered to be a potential source, the basis for the increased activity of acid phosphatase but not of β-glucuronidase might be explained in terms of differential permeability (S3) or of the heterogeneous nature of lysosomes (R1).

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NORMAL AND ABNORMAL HUMAN HEMOGLOBINS

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1. Introduction

In 1963, this author summarized in these Advances under the same title the topic of the heterogeneity of human hemoglobin and discussed the structural, physicochemical, and functional aspects of the normal hemoglobins and some of their variants (H36). During the past nine years the study of these normal and abnormal proteins has advanced greatly and has provided insight into the structure and function of normal and abnormal hemoglobins, and in the genetic control of protein synthesis. Recognition of various abnormalities in the synthesis of hemoglobin has led to a better understanding of mechanisms which can cause disease.

This review deals in detail with recent advances; more important early observations, i.e., those made prior to 1963, are only briefly mentioned. No attempt is made to review the various topics completely, and it is, therefore, suggested that the following review articles and monographs be consulted:

a. General reviews (B36, C24, H33, H37, J20, J25, J26, J27, J28, L10, L13, M8, N9, R29, R36, S15, S44, S66, W20)
b. Thalassemia (C24, F8, F9, M30, W9)
c. Distribution (J28, L30)
d. Structure and function (B4, B67, H26, H27, H52, R30, R33, S15, S20, W25)
e. Genetics and synthesis (B8, H52, I3, N9, S55, W14, Z2, Z3)
f. Embryonic and fetal hemoglobin (H30, H52, K13)
g. Methodology (C14, J29)

In addition, references to articles reviewing specific aspects of the topic to be discussed are made at appropriate places in the text.

2. Normal Human Hemoglobins

2.1. Hemoglobins of the Adult, the Newborn, and the Fetus

2.1.1. *The Normal Types*

In man, a number of different hemoglobin polypeptide chains are synthesized from the time of conception to adult life. The two adult hemoglobins, Hb-A or $\alpha_2\beta_2$ and Hb-A_2 or $\alpha_2\delta_2$, are first observed in minor amounts in the embryo during the second trimester of pregnancy. After birth they rapidly replace fetal hemoglobin (Hb-F or $\alpha_2\gamma_2$), and almost no Hb-F is present in a 6-month-old infant. The Hb-F is the

major hemoglobin of the newborn and of the fetus; it is a heterogeneous protein because two chemically different γ chains, termed $^{G}\gamma$ and $^{A}\gamma$, have been recognized (S22). This heterogeneity is genetically determined (see Section 5).

The possible existence of embryonic hemoglobins was first suggested in 1954 (D10). Some investigators (H1, H2, Z1) found electrophoretic and chromatographic differences between the hemoglobin of fetuses of 10- to 20-week pregnancies and that of the newborn, but others (M13) were unable to confirm this. However, two distinct hemoglobin types, termed Hb-Gower 1 and Hb-Gower 2, have been observed in all young embryos up to a length of about 7 cm and not older than 12 weeks (H29, H30, H31, H32, K13, K15, K16). The structures of these embryonic hemoglobins are ϵ_4 and $\alpha_2\epsilon_2$, respectively; the ϵ chain is probably the first hemoglobin chain which is synthesized during embryonic development.

A third embryonic hemoglobin, termed Hb-Portland 1, has recently been described (C1, C2, H6, T8, W17). The protein was first discovered in a malformed newborn with a complex autosomal chromosomal mosaicism. However, it is probably also present for some 0.5 to 5% in newborn infants with a D_1 trisomy and in very small amounts (0.1–0.2%) in normal newborns. Hb-Portland 1 does not contain α chains but is composed of one pair of γ chains and one pair of chains which have been termed ζ chains. The ζ chain is probably a normal embryonic hemoglobin chain. Hb-Portland 1 does react with free α chains to form Hb-F or $\alpha_2\gamma_2$ and a $\alpha_2\zeta_2$ component (T8). It seems, therefore, that the ζ chain can combine with the α chain, but the affinity of the α chain for the ζ chain is apparently lower than for either the ϵ chain or γ chain. Embryonic hemoglobins have also been found in several mammalian species, such as the pig, cattle, and sheep (K13, K15).

2.1.2. *The Primary Structures*

The amino acid sequences of the α and β chains were determined in the early 1960's [for references, see (B4)]. The α chain contains 141 amino acids and the β chain 146 (the γ and δ chains are also 146 amino acid residues long). Figures 1 and 2 give the sequences of the α chain and the β chain, respectively.

The analysis of the primary sequence of the γ chain from cord blood was completed in 1963 (S19). The 17 differences between the amino acid compositions of the β chains and the γ chains express themselves in 39 differences in sequence; the positions where the differences are observed are located in various areas of the chains. The four isoleucyl residues, a distinct characteristic of this fetal protein, are found in positions 11,

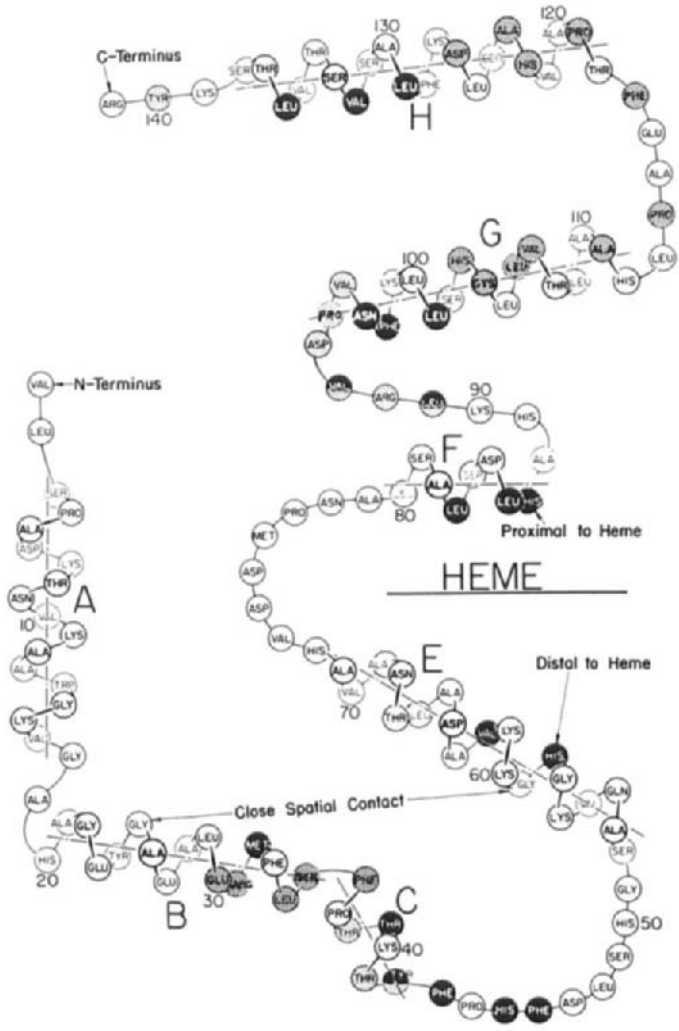

FIG. 1. Two-dimensional presentation of the spatial arrangement of the amino acid residues of the α chain of human hemoglobin. ● Residues in contact with the heme group. ◉ Residues that participate in the $\alpha_1\beta_1$ contact. ◎ Residues that participate in the $\alpha_1\beta_2$ contact.

54, 75, and 116. The two types of γ chain differ at a minimum in one position, namely position 136, which is occupied by a glycyl residue in the $^{G}\gamma$ chain and by an alanyl residue in the $^{A}\gamma$ chain (S22). Fetal hemoglobins with either $^{G}\gamma$ or $^{A}\gamma$ chains cannot be separated from each other by electrophoresis or chromatography.

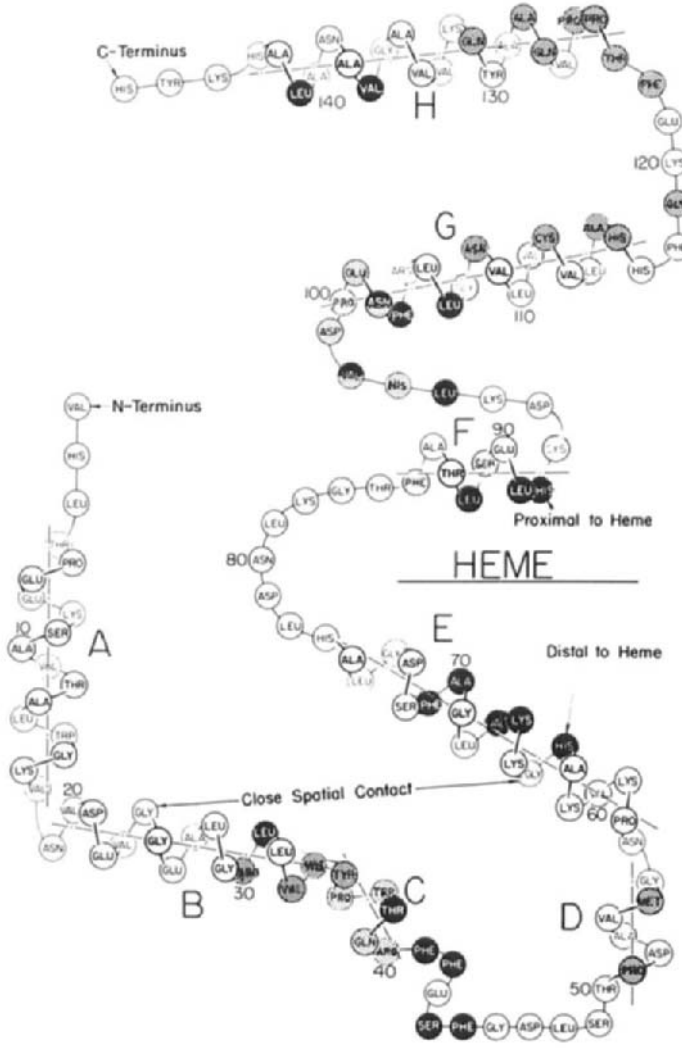

FIG. 2. Two-dimensional presentation of the spatial arrangement of the amino acid residues of the β chain of human hemoglobin A (see also Fig. 1).

The primary structure of the δ chain differs from that of the β chain in 10 residues, and the following replacements are observed: Ser-9 by Thr; Thr-12 by Asn; Glu-22 by Ala; Thr-50 by Ser; Ala-86 by Ser; Thr-87 by Gln; His-116 by Arg; His-117 by Asn; Pro-124 or -125 by Gln; and Val-126 by Met (S20). (Numbers denote positions in the chain.)

The primary structure of the ϵ chain is not established, and only results of preliminary structural analyses have been reported (H30, K15). Extensive studies of the ζ chain have revealed rather unique structural features (C2). Its amino acid composition, for instance, differs from those of all other hemoglobin chains and is, among others, characterized by the presence of five isoleucyl residues. Tryptic hydrolysis produced several peptides with amino acid compositions not observed for any of the tryptic peptides of the α, β, γ, or δ chain, although other peptides had compositions identical to those of tryptic peptides of the γ chain. There seems to be no doubt that the structures of the ϵ and ζ chains are different (C2).

2.1.3. *The Three-Dimensional Structure*

The brilliant observations by Perutz and collaborators (B45, B46, C36, C37, M31, M32, M33, P5, P6, P7, P8, P10, P11, P12, P13, P15, P16) have given us a detailed insight in the secondary, tertiary, and quaternary structures of the hemoglobin molecule. The first X-ray crystallographic data were obtained at low resolutions and made the general course of the polypeptide chains apparent whereas the latest X-ray data at high resolutions present detailed indications of atomic positions.

About 80% of both the α chain and the β chain is in a conformation of a right-handed α helix. There are eight α helices in the β chain which are labeled A, B, C, D, E, F, G, and H in order from the amino terminus. Seven α helices are present in the α chain because the D helix is missing (Figs. 1 and 2). Intrahelical segments are present between all but a few helices. These nonhelical parts are denoted by the helices at either end—for instance, EF. The spatial arrangements of the α chain and of the β chain are closely similar despite some significant differences. It is noteworthy that many prolyl residues in the two chains occur in second position of a helix. The first position in such a helix is usually occupied by an aspartyl, or an asparaginyl, or a threonyl residue which forms a hydrogen bond with the main chain imino group of the fourth residue. Such an arrangement generally leads to a change in the direction of the main chain.

Polar residues are almost completely absent in the interior of the α and the β chain (exceptions are an occasional threonyl and seryl residue). The nonpolar side chains are found in the interior, in crevices at the surface, and at the boundaries of unlike chains; only a few nonpolar side chains are found at the surface protruding into the surrounding water.

The heme group lies in a nonpolar pocket of each chain. There are

approximately 60 interactions between atoms of the polypeptide chain and of the heme group; almost all are nonpolar. The residues of the α chain and of the β chain which participate in this heme contact are depicted in Figs. 1 and 2. Thus, the ferrous iron of the heme group is in a nonpolar, hydrophobic, environment which facilitates greatly its ability to combine reversibly with molecular oxygen. Of the six positions of the iron in heme, four are occupied by the pyrroles of porphyrin and one by the nitrogen atom of the imidazole ring of the proximal histidyl residue (α-87 and β-92) whereas the sixth position is empty in deoxyhemoglobin or is liganded as, for instance, with oxygen in oxyhemoglobin. This position is in the close vicinity of the distal histidyl residue (α-58 and β-63).

Contacts between unlike chains are of two different kinds; the $\alpha_1\beta_1$ contact is more extensive than the $\alpha_1\beta_2$ contact. Thirty-four residues, involving about 110 atoms, participate in the $\alpha_1\beta_1$ contact (Figs. 1 and 2). The majority of the interactions are nonpolar; there are five hydrogen bonds, which are all in contact with water. Examination of the two figures will fail to show the actual contacts between the two chains. However, in essence, the B helix of the α chain is in contact with the H helix of the β chain and vice versa, whereas other contacts involve the α-G helix and the α-GH interhelical section with the β-G helix.

The $\alpha_1\beta_2$ contact involves 19 residues contributing about 80 atoms (Figs. 1 and 2). There are two hydrogen bonds, one between the side chains of Asp G1 (94) α and of Asn G4 (102) β and a second between the side chains of Thr C6 (41) α and His FG4 (97) β. All other interactions are nonpolar. All residues participating in the contact are in helices C and G, and in the FG segment except for residue Tyr 140 α.

In solution, the hemoglobin tetramer is in equilibrium with the $\alpha\beta$ dimer. This dissociation is dependent on several factors, such as the ligand at the iron atom, the hemoglobin concentration, the electrolyte concentration, the pH, and the temperature. This symmetrical dissociation into dimers ($\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$) occurs at the $\alpha_1\beta_2$ contact as was recently again demonstrated by Smith *et al.* (S54) in their studies of two α chain variants in which one of the residues of the $\alpha_1\beta_2$ contact, Pro G2 (95) α, was replaced by Leu and Ser, respectively. Dissociation at the $\alpha_1\beta_1$ contact leading to the formation of α and β chain monomers occurs only at pH values below 4.9 or above 11.0.

Contacts between like chains consist mainly of salt bridges involving the terminal residues.

Of the 39 differences between the β and γ chains, 2 (in positions 70 and 71) are part of the heme contact and 4 (in positions 51, 112, 116, and 125) are part of the $\alpha_1\beta_1$ contact. The absence of replacements of

residues participating in the $\alpha_1\beta_2$ contact explains the comparable dissociation rates of the hemoglobins A ($\alpha_2\beta_2$), F ($\alpha_2\gamma_2$), and also of A_2 ($\alpha_2\delta_2$), into asymmetric dimers (the $\alpha_2\gamma_2 \rightleftarrows 2\alpha\gamma$ reaction). The four replacements in the $\alpha_1\beta_1$ contact of Hb-F, however, are probably responsible for the relatively slow dissociation at this contact (the $\alpha\gamma \rightarrow \alpha + \gamma$ reaction) thus explaining the resistance of Hb-F against alkali and the slow rate of hybridization at acid pH (H35). The substitution of tyrosyl residue in position 130 of the β chain by a tryptophanyl residue in the γ chain accounts for the well-known difference in ultraviolet spectral absorption between the hemoglobins A and F.

2.1.4. *The Oxygenation-Deoxygenation Reaction*

Early observations have shown that in the transition of the oxy form of hemoglobin to the deoxy form the movement in the $\alpha_1\beta_1$ contact is slight in comparison with that in the $\alpha_1\beta_2$ contact. This $\alpha_1\beta_2$ contact is apparently constructed in such a way that the two subunits are able to slide past one another. Since the $\alpha_1\beta_2$ contact is closely connected with the heme groups (Figs. 1 and 2), any change in this contact area might well influence the environment of the heme. The effect of oxygenation is also illustrated by the alteration in distances between the iron atoms (Table 1). Most striking is the change in the distance between the iron atoms of the two β chains, and small variations are also apparent in the $\alpha_1\beta_1$ distance for iron. These changes have been viewed in light of the interaction between heme groups, a phenomenon which gives the oxygen dissociation curve its characteristic shape. *Heme–heme interaction* has often been considered to be an allosteric reaction, suggesting that the oxygenation of one heme group leads to such structural alterations of the entire molecule that the oxygenation of the remaining heme groups is greatly facilitated.

More recently Perutz (P10) has proposed a stereochemical mechanism of heme–heme interaction which describes in detail the cooperative effects in hemoglobin. Most important in the oxygenation–deoxygenation

TABLE 1
DISTANCES BETWEEN IRON ATOMS IN OXYHEMOGLOBIN AND IN DEOXYHEMOGLOBIN

Distance	Oxy (Å)	Deoxy (Å)	Difference (Å)
β_1–β_2	33.4	39.9	+6.5
α_1–α_2	36.0	34.9	−1.1
α_1–β_1(α_2–β_2)	35.0	36.9	+1.9
α_1–β_2(α_2–β_1)	25.0	24.6	−0.4

mechanism is the substantial movement of the iron atom relative to the porphyrin ring. X-ray studies of deoxyhemoglobin have indicated that the iron is about 0.8 Å out of plane of the four nitrogen atoms of the porphyrin whereas in oxyhemoglobin the iron atom lies (almost) exactly in the plane. Thus, the reaction with oxygen will cause a shift which is sufficient to force drastic changes in various contacts leading to cooperative movements in the hemoglobin molecule. These changes within the α and β chain subunits concern a movement of the F helix toward the center of the molecule and toward the H helix (Fig. 3). The pocket between helices F and H is narrowed and can no longer accommodate the aromatic side chain of the penultimate tyrosyl residue (Tyr 145 β and Tyr 140 α). When this side chain is expelled from its crevice, it pulls the carboxyl terminal residue with it. This causes a gradual rupture of salt bridges between the two α chains [between the carboxyl group of Arg HC3 (141) α_1 and the α amino group of Val NA1 (1) α_2, and between the guanidinium group of Arg HC3 (141) α_1 and the carboxyl group of Asp H9 (126) α_2] and between the α and the β chains [between the carboxyl group of His HC3 (146) β_1 and the ϵ-amino group of Lys C5 (40) α_2, and between the imidazole group of His HC3 (146) β_1 and the γ carboxyl group of Asp FG1 (94) β_1]. The result is the removal of a constraint that was holding the polypeptide in an arrangement characteristic for the deoxy structure. In the new conformation the oxygen affinity is increased and the successive oxygenation of subunits leads to a concurrent shift in contacts between the α and β chains.

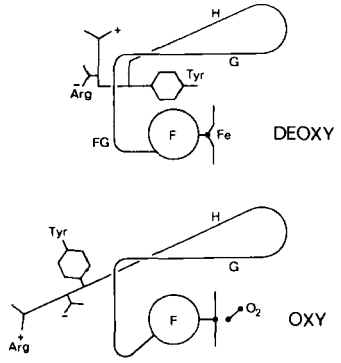

FIG. 3. Diagrammatic sketch showing the change in tertiary structure of a hemoglobin α chain on reaction with oxygen. Movement of the iron atom into the plane of the porphyrin ring causes a movement of helix F toward helix H, which expels tyrosine in position 140 from its pocket between the two helices. From (P10), M. F. Perutz, Stereochemistry of cooperative effects in haemoglobin. *Nature* (*London*) **228**, 726 (1970) with permission of the author and publisher.

This shift of the quaternary structure results in a more rapid oxygenation of the remaining iron atoms, a widening of the heme pockets, and a complete rupture of all salt bridges involving the carboxy-terminal residues. Perutz (P10) suggests that the subunits react in the order of α_1, α_2, β_1, β_2 but the mechanism does not depend on a specific sequence.

2.1.5. *The Mechanism of the Bohr Effect* (K10, K11, P9, P17, P18, T9)

The Bohr effect, i.e., the uptake of hydrogen ions above pH 6 (known as the alkaline Bohr effect), is physiologically important because the H^+ ions released on uptake of CO_2 by the blood from the tissues are neutralized through this mechanism. Figure 4 illustrates the Bohr effect of normal hemoglobin; the reverse effect, the acid Bohr effect, has no physiological significance because it is observed below pH 6.

The alkaline Bohr effect is synchronous with the oxygenation reaction and the liberation of Bohr protons is exactly proportional to the amount of oxygen taken up. The effect also results from specific changes within the hemoglobin molecule and the latest data indicate that 50% of the effect is due to the salt bridge between the imidazole side chain of His

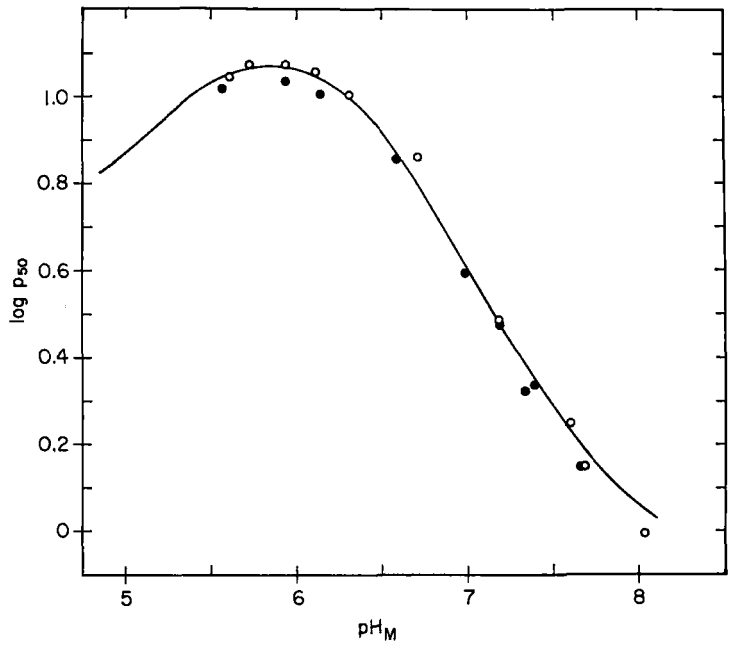

FIG. 4. The Bohr effect of human hemoglobin. Measurements were made at 25°C in 0.01 *M* phosphate; hemoglobin concentration: 0.2 g/100 ml. From Tomita and Riggs (T9) with permission of the author and publisher.

HC3 (146) β and the γ-carboxyl side chain of Asp FG1 (94) β whereas similar bridges between α-amino groups of the amino-terminal valyl residues and the α-carboxyl group of the carboxy-terminal arginyl residues are responsible for the remainder of the effect.

The acid Bohr effect is attributed to a decrease in pK of conjugate bases from 5.7 in oxy to 4.9 in deoxyhemoglobin.

2.1.6. *Hemoglobin and 2,3-Diphosphoglycerate (2,3-DPG)*

The observation by Chanutin and collaborators (C10, S64) and by Benesch and Benesch (B25, B26) that 2,3-DPG (and also ATP and inositol hexaphosphate, or IHP) binds to hemoglobin resulting in a shift to the right of the oxygen dissociation curve has led to numerous reports which describe topics related to biochemical and medical aspects of the 2,3-DPG and hemoglobin relationship. These papers have been adequately summarized (B71, H52).

2,3-DPG is present in the human red cell in a molar ratio slightly in excess of hemoglobin. This organic phosphate binds to deoxyhemoglobin and has a greatly decreased affinity for liganded forms. The binding of 2,3-DPG to deoxyhemoglobin is decreased by an increase in molarity of salt, in pH, and in temperature. The binding is in a ratio of 1 mole of 2,3-DPG to 1 mole of deoxy Hb. Because 2,3-DPG also binds to hemoglobin H or β_4, the binding site is on the β chain. Although not definitely established, the binding sites on this chain likely are Val NA1 (1), Lys EF6 (82), and His H21 (143). Apparently one 2,3-DPG molecule fits between two β chains of the deoxy configuration and are kept in place by hydrogen bonding between amino and/or imidazole groups of these residues and the charged groups of the organic phosphate. In this way, it provides four additional salt bridges crosslinking the β chain; these extra bridges stabilize the quaternary structure of deoxyhemoglobin. Upon oxygenation, the distance between the α-amino groups of Val NA1 (1) increases from 16 Å to 20 Å so that no contacts can be made with the phosphates of 2,3-DPG. Moreover, the H helices in two β chains close up and the 2,3-DPG molecule is expelled from the central cavity (P9).

The importance of the amino terminal valyl residue for the binding of 2,3-DPG is indicated by the observation that hemoglobins with blocked amino terminal residues, such as the minor human hemoglobin A_{Ic} (B77) and one of the hemoglobins of the cat (T1), and hemoglobins that lack this residue and thus have β chains with only 145 residues, such as the hemoglobin of sheep (B79), do not combine with this organic phosphate.

Figure 5 presents a diagrammatic sketch of the deoxyhemoglobin mole-

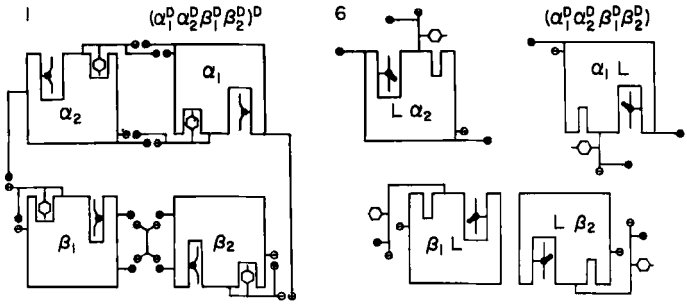

FIG. 5. Diagrammatic sketch showing differences in quaternary structure between deoxyhemoglobin and oxyhemoglobin. (*1*) Deoxyhemoglobin with all salt bridges intact and with one molecule of 2,3-DPG clamped between the β chains. (*6*) Oxyhemoglobin without salt bridges and 2,3-DPG. Note also that the penultimate tyrosyl residue is expelled from the pocket. From Perutz (P9) with permission of the author and publisher.

cule with salt bridges between the terminal residues, the 2,3-DPG residue between the two β chains, and the side chains of the penultimate tyrosyl residues in the appropriate pockets, and of the fully oxygenated hemoglobin molecule without the salt bridges and the 2,3-DPG residue, and with the tyrosyl residues expelled from the pockets. The figure is taken from reference P9; consultation of this reference will further illustrate the changes in quaternary structure during the oxygenation–deoxygenation process.

2.2. Minor Hemoglobins

2.2.1. *The Types in the Adult*

A hemoglobin fraction which migrates more rapidly than hemoglobin A during electrophoresis at pH 8.6 was observed some 15–20 years ago (K26, K27, K28). Chromatography on columns of Amberlite IRC-50 (A11, C21, J12, S13), CM-cellulose (H40, H41, M15), and CM-Sephadex (D9) has resolved this fraction into many minor components. Figure 6 illustrates chromatograms obtained by CM-Sephadex chromatography; at least four, and probably five, minor zones precede the major Hb-A fraction. The hemoglobins in zones III and IV have been studied most extensively; these components are the same as the minor hemoglobins A_{Ic} and A_{Id} which have been isolated by Amberlite IRC-50 chromatography.

2.2.1.1. *The Nature of Hb-A_{Ic}.* Earlier studies by Schroeder and collaborators (H18, H19, H20) identified this component as a condensation product, a Schiff base, between one molecule of Hb-A and one mole-

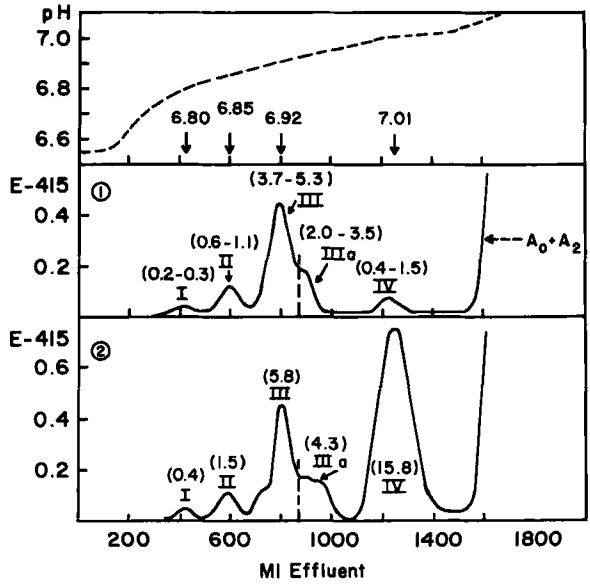

FIG. 6. Separation of minor hemoglobin components by chromatography on columns of CM-Sephadex. (*1*) A freshly prepared hemolysate. (*2*) Same hemolysate, aged for 40 days. The values in parentheses represent the relative amounts of the individual zones; the elution pH value of each individual zone is given at the top of the first chromatogram.

cule of a ketone or aldehyde with the amino terminus of one of the two β chains as the point of linkage (Hb-A_{Ic} = $\alpha_2\beta\beta^{R=0}$). Recent studies (B52, E2) have shown that the amino terminal residues of both β chains are blocked (Hb-A_{Ic} = $\alpha_2\beta_2^{R=0}$). Bookchin and Gallop (B52) identified the compound as a hexose. Hb-A_{Ic}, therefore, is a glycoprotein but appears to be unique in the mode of attachment of the sugar to the protein. Schroeder and Holmquist (S21) have speculated about its function, but no definite information suggesting a specific role of this hemoglobin component inside the red blood cell is at present at hand.

The quantity of Hb-A_{Ic} is rather constant at 4–6% in normal adults and in patients with various hematological disorders (Fig. 7); a significant decrease is evident in patients with iron deficiency anemia (H24). A 2-fold increase in Hb-A_{Ic} or in an A_{Ic} like component is also observed in patients with diabetes (H43, R5, R6, T10). This glycohemoglobin may be different from the normally occurring Hb-A_{Ic} because the most recent studies suggest that an amino sugar is blocking the amino terminus (R5). The physiological significance of the component is not clear.

2.2.1.2. *Minor Hemoglobins and Glutathione.* Incubation of red cell hemolysates with oxidized glutathione produces a component (Hb-A_{Id}) with a unique chromatographic mobility; the same minor hemoglobin fraction is formed upon aging of hemolysates (H43, H45, H46). Its structure is $\alpha_2(SH)_2 \cdot \beta_2(SSG)_2$ indicating that one of the two sulfhydryl groups in each of the two β chains (namely that in position 93) is blocked by a glutathione residue (H46). The formation of this mixed disulfide can be prevented with reducing agents, including reduced glutathione. Hb-A_{Id} has an increased oxygen affinity and a decreased heme-heme interaction. The formation of this minor hemoglobin *in vivo* is negligible (B58).

2.2.1.3. *Minor Hemoglobins and 2,3-Diphosphoglycerate.* A complex between hemoglobin and 2,3-DPG has been demonstrated by moving boundary electrophoresis in cacodylic acid–sodium cacodylate buffer, pH 6.5 (B32, S64). Attempts to demonstrate a similar component by chromatographic means have failed because the organic acid binds preferentially deoxyhemoglobin which is difficult to analyze by chromatography. Moreover, it has been shown that the pH is an important factor in the separation of 2,3-DPG from Hb. The bond between the two compounds is easily broken when the pH is raised above 7 (B31); most chromatographic procedures utilize developers with pH values of around 7 or above.

2.2.1.4. *Other Minor Hemoglobins.* The nature of Hb-A_{Ia} and Hb-A_{Ib} has not been clarified. The two minor hemoglobins are present in decreased quantities in patients with hemolytic anemia, and in increased amounts in patients with myelocytic leukemia and iron deficiency anemia (Fig. 7) (H24). An unknown, fast moving, minor hemoglobin fraction has been observed when red cell hemolysates from children with elevated blood lead levels were analyzed by electrophoresis (C11).

2.2.2. *Hb-F_1, the Minor Hemoglobin of the Newborn*

Allen *et al.* (A11) first separated a minor Hb-fraction, termed Hb-F_1, from umbilical cord blood; the ratio between this F_1 and the major F_0 fraction is about 1 to 10 and remains constant during most of fetal development (M13). Its structure has recently been established (S17, S62), and it seems that the difference between the two fetal hemoglobins concerns the amino-terminal residues of the two γ chains which are acetylated in the minor hemoglobin component (Hb-F_0: $\alpha_2\gamma_2$; Hb-F_1: $\alpha_2\gamma_2^{acetyl}$). No information is at present available suggesting a mechanism by which these acetyl groups are introduced. The functional significance of the Hb-F_1 fraction is also not clear; it has been suggested that the function

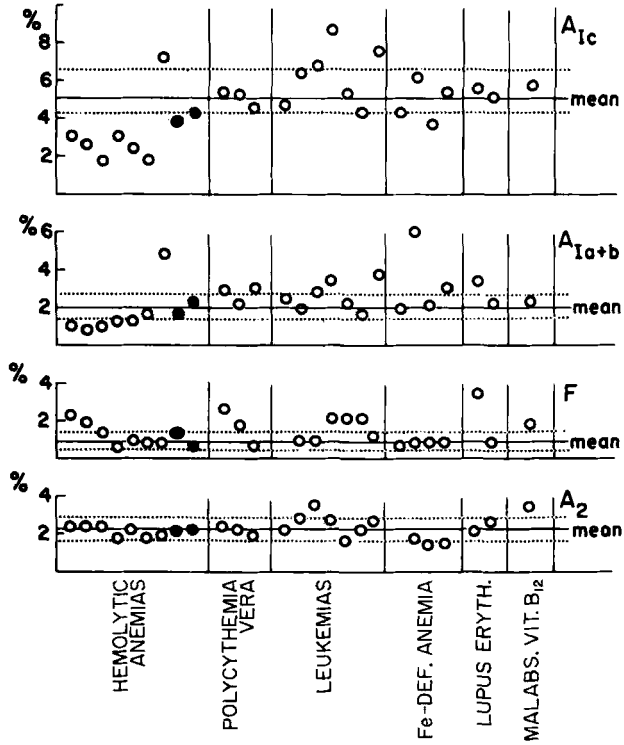

FIG. 7. The minor hemoglobin components of patients with various hematological disorders. From left to right: hemolytic anemias, polycythemia vera, leukemias, iron-deficiency anemia, lupus erythematosus, malabsorbed vitamin B_{12}. ●, Post splenectomy. From Horton and Huisman (H24) with permission of the authors and publisher.

of hemoglobins with blocked amino terminal residues is related to the carriage of CO_2 (K10).

2.3. Genetic Control of Hemoglobin Synthesis

2.3.1. *Structural Loci*

The synthesis of each hemoglobin polypeptide chain is under separate genetic control. Evidence to this effect has been obtained from studies of critical families with (combinations of) specific hemoglobin variants; these data are extensively reviewed by Huehns and Shooter (H33). Thus, separate loci exist directing the synthesis of α, β, γ, δ, ϵ, and ζ chains. All data, including data from complete structural analyses (S18), in-

dicate that the α chains of the hemoglobins A, F, A_2, and probably also of Hb-Gower 2, are the same and arise from a common pool. During development this pool is inadequate to supply the non-α chains with the necessary α chains to form the tetrameric molecule consisting of two pairs of different polypeptide chains (H35).

Preliminary genetic linkage analysis for the Hb_β and Hb_δ loci with genetic loci determining 17 other polymorphic systems has suggested linkage with the Duffy locus, which is closely linked to a polymorphic secondary constriction on one of the arms of chromosome 1 (N7). This study is the first to give an indication for the localization of hemoglobin loci on a specific chromosome.

There seems to be no doubt that the Hb_β and Hb_δ loci are linked; this is based on studies of families with β and δ chain variants in which the two abnormalities segregate without cross-overs (B59, C8, H23, P3, R9), on that of a family in which the Hb_δ locus is linked to that for β-thalassemia (H42), and on the primary structures of the Lepore hemoglobins (see Section 3.1.8). Studies of four families in which mutants of the Hb_α locus and of the Hb_β locus are segregating have shown the absence of linkage (A14, L28, M14, R13, W12). The possible linkage between the Hb_γ loci and the Hb_β and Hb_δ loci is discussed in Section 5.

2.3.2. *Duplicated Structural Loci*

Duplicated Hb_α loci have been found in many mammalian species [for reviews, see (C16, H47, H52)]. Evidence for a similar phenomenon in man (or at least in some families) is forthcoming from studies of Brimhall *et al.* (B73) and possibly from analyses of subjects with various forms of α-thalassemia (see Section 4.6). However, failure to find Hb-A in subjects with a homozygosity for the α chain variant Hb-J-Tongariki (A1) or in double heterozygotes for the α chain variant Hb-Q and α-thalassemia type-1 (D7, V6) suggests the presence of a single Hb_α locus in these families. Analyses of additional families might clarify this confusing situation.

Multiplicity of the γ chain structural locus has been established (S22); at least two loci, designated the Hb_{A_γ} and Hb_{G_γ} loci, control the two types of γ chains which differ at a minimum in one position (position 136). The genetic heterogeneity of Hb-F is reviewed in Section 5.

Multiplicity of the Hb_β locus does not exist because homozygotes for many β chain variants do not synthesize normal β chains; this conclusion is supported by mounting chemical evidence (V7).

The scheme of Fig. 8 summarizes eight hemoglobin structural loci, their polypeptide chain products, and the various hemoglobin types which are found in the human at different stages of development.

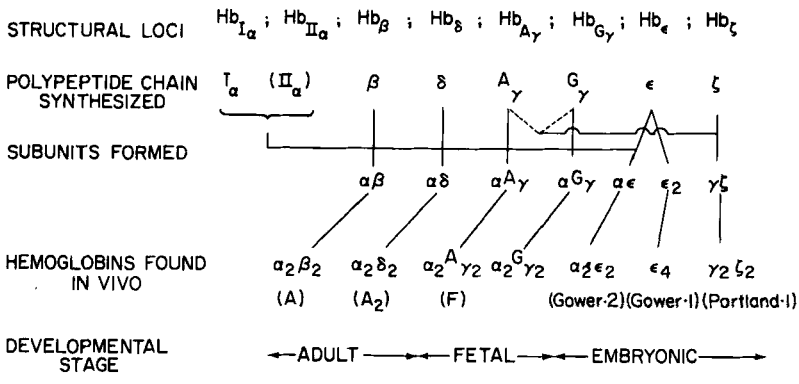

FIG. 8. Genetic regulation of the synthesis of human hemoglobins.

2.3.3. *Rate-Controlling Mechanisms*

During development, the synthesis of embryonic hemoglobin polypeptide chain(s) is succeeded by that of the γ chains and at a later stage by that of the β and δ (Fig. 9). The ontogeny of these hemoglobin chains is thoroughly reviewed by Kleihauer (K15), and Fig. 9 is based on information taken from that reference. Hb-Gower-1, or ϵ_4, is the major

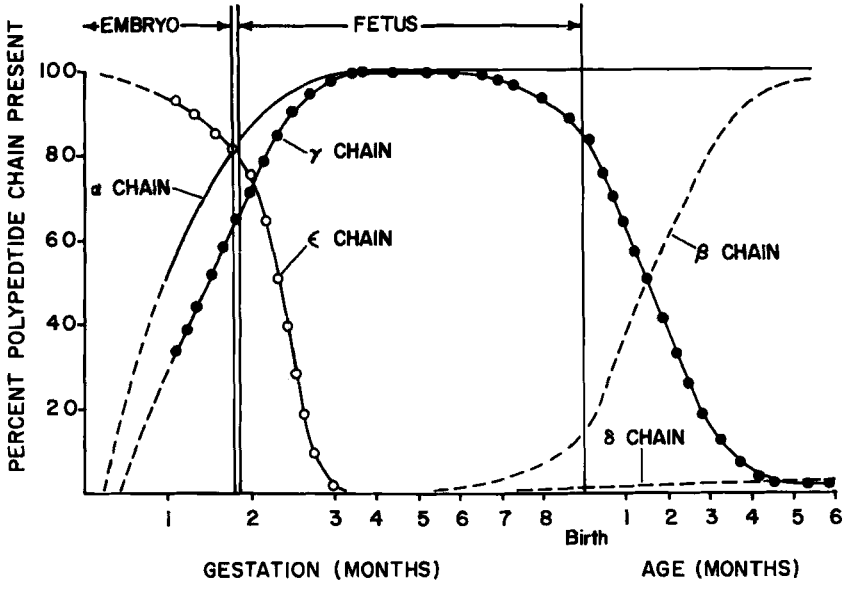

FIG. 9. Relative amounts of hemoglobin polypeptide chains in different stages of development in man. Data concerning the ζ chain are not included.

hemoglobin of the embryo. The apparent α chain deficiency of the very early embryo decreases gradually but small amounts of Hb-Portland-1, or $\gamma_2\zeta_2$, and Hb-Bart's, or γ_4, are often present in the blood of normal newborns. The formation of Hb-Gower-2, or $\alpha_2\epsilon_2$, in embryos who at that stage do not yet produce γ chains, suggests that the onset of the α chain synthesis precedes that of γ chains.

What is the mechanism controlling the rate of synthesis of each of these hemoglobin chains during development? It has been suggested that the site of red cell production is a major factor; however, β (and δ) and γ chains can be synthesized by red cell precursors in the bone marrow as well as in the liver (F11, T4) whereas the presence of small amounts of Hb-Gower 2 (or $\alpha_2\epsilon_2$) and of Hb-Portland-1 (or $\gamma_2\zeta_2$) in (some) cord blood samples (C2, H22) show that red cell precursors in sites other than in the metamorphosing embryo are able to synthesize these polypeptide chains.

Regulatory genes which control the rates of synthesis of various polypeptide chains have been suggested (M29, N11, Z3); this concept is based on work in bacterial genetics (J2). At present, no conclusive evidence has been put forward to indicate that such controller genes are operative in the hemoglobin synthesizing system. However, some strong indication for the existence of a comparable regulatory mechanism in Caprini species comes from recent studies indicating the replacement of the normally occurring Hb-A (or $\alpha_2\beta_2{}^A$) by Hb-C (or $\alpha_2\beta_2{}^C$) during anemic states, and vice versa (G3, H48, M26, V2). The biological switch of the $Hb_{\beta A} \rightleftarrows Hb_{\beta C}$ structural genes is influenced by humoral factor(s) which either inhibit or stimulate this mechanism; one factor might be identical to the erythropoietic stimulating factor (ESF) (B62, L16). It is possible that the action of this factor is based on becoming part of an activator–inhibitor complex with an operator–regulator mechanism which controls the rates of synthesis of the $Hb_{\beta A}$ and $Hb_{\beta C}$ structural genes.

2.4. The Biosynthesis of Hemoglobin

The synthesis of hemoglobin has been studied *in vitro* in bone marrow and reticulocyte preparations using labeling techniques and short-term incubations. The process has recently been reviewed by Weatherall and Clegg (W14); this review should be consulted for additional references. Figure 10 summarizes the general organization of the synthesis of hemoglobin.

2.4.1. *Chain Synthesis*

Synthesis of α and β chains occurs on groups of ribosomes (containing 4–6 ribosomes) which become attached to mRNA. This mRNA carries

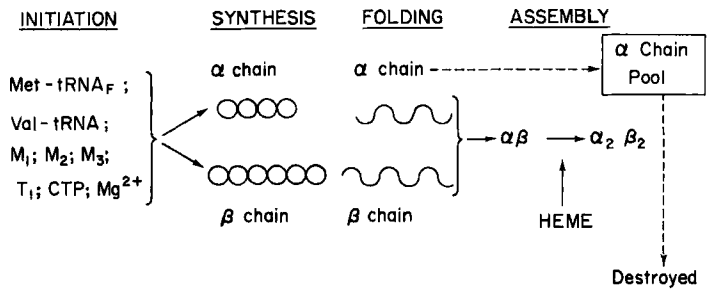

FIG. 10. The biosynthesis of hemoglobin α and β polypeptide chains.

the genetic information derived from the DNA of the gene. The rates of translation of mRNA for the α and β chains are slightly different; the α chain is translated faster than the β chain (H64), but a slight excess of mRNA for β chains seem to be present (H65). Thus, the two classes of mRNA are translated within the same cell at slightly different rates and a slight excess of free α chains results. This α chain pool can be found in the reticulocyte. Excess α chain is destroyed by unknown means so that it is not present in the mature red cell. The polysomes which are the site of synthesis of the β chains are larger than the polysomes on which the α chains are synthesized.

2.4.2. *Chain Initiation and Termination*

The mechanism responsible for the initiation of peptide chain synthesis has been studied in bacteria [for review, see (L14)]. The first step appears to be the binding of *N*-formylmethionyl tRNA (fMet-tRNA$_f$) at a unique initiating codon (GUG) in the mRNA. At least two species of methionyl-tRNA are known; one, known as Met-tRNA$_f$, can be formylated but the second (Met-tRNA$_m$) recognizes only the internal AUG codon and thus incorporates methionyl residues into the polypeptide chain at internal positions.

Increasing evidence suggests that Met-tRNA$_f$ is also the initiator tRNA in eukaryotic systems (C35, G16). The failure of previous experiments to demonstrate the role of this Met-tRNA$_f$ for the *in vitro* protein synthesis is probably due to the lack of protein initiation factors, M_1, M_2, and M_3, which are present in a ribosomal salt-wash protein fraction (P24, S35, S36). The most recent experiments by Anderson and coworkers (C34, C35) show that the Met-tRNA$_f$ binds the initiation factors M_1 and M_2 to form an initiation complex with messenger RNA. The binding of this complex requires CTP and Mg^{2+} ions. A methionyl-valine dipeptide production is the next step in the biosynthesis of the chain; the synthesis of this bond requires Mg^{2+} ions, an additional initiation

factor M_3, Val-tRNA, and an elongation factor T_1. Other amino acyl-tRNA's are unable to replace the Val-tRNA to form a peptide bond with methionine. It is of interest that the β chain of several mammalian hemoglobins carry a methionyl residue in amino terminal position [for review, see (K12)].

The peptide chain grows in length by the addition of amino acid residues from the amino-terminal end (D5). The assembly time of one chain is estimated at 15–30 seconds at 37°C. Termination of the chain assembly is probably due to the presence of the mRNA codon UAA (or the less likely codons UAG and UGA) which is read as end chain and causes the chain to be detached from the assembly line.

Upon release of the two types of chain, a folding of the polypeptides occurs to obtain a stable physicochemical configuration. The assembly of an $\alpha\beta$ unit rather than an α_2 or β_2 dimer is also based on favorable steric arrangements. There is evidence that the heme–globin association occurs after the release of the completed chain from the ribosomes and probably after the formation of the $\alpha\beta$ dimer. The formation of a stable tetramer follows soon after the heme–protein association has taken place.

3. Hemoglobin Abnormalities

Since the discovery of the first hemoglobin variant (Hb-S) by Pauling, Itano, Singer, and Wells (P1) over 150 additional abnormal hemoglobins have been described. These variants can be abnormal in α, β, γ, and δ chains, and specific structural aspects of some other abnormalities place these in special categories. This review attempts to list all variants of which the structural abnormalities have been reported; the survey of the literature was completed August 31, 1971. The usefulness of such a list may be limited because it likely will be out of date before it is prepared for printing. The list is divided over several tables which are arranged in such a way that they will guide the reader through the labyrinth of variants.

3.1. Variants with Abnormalities in Either α or β Chains

Tables II, III, IV, and V list abnormal hemoglobins which differ from the normal by the replacement of one amino acid residue by another at a specific position in either the α or the β chain. The total number is 132. Each of these hemoglobin types is produced because of a variation, by mutation, of the hemoglobin chain structural locus governing the synthesis of the specific polypeptide. The amino acid substitutions found in all these variants can be explained by the change of one nucleotide of the triplet which codes for the original residue.

The recent construction of atomic models of the oxyhemoglobin molecule and of the deoxyhemoglobin molecule, based on X-ray analyses at

high resolution (see Section 2), offers an unique opportunity to study the effect of specific amino acid substitutions on the physicochemical and/or functional properties of the protein. Data from these analyses have subdivided the 132 variants into different groups of abnormalities—for instance, the unstable hemoglobins that are one of the causes of congenital hemolytic anemia and variants that may affect its carrier because of abnormal oxygen dissociation properties. These abnormalities as well as homozygous sickle cell disease and other sickle cell syndromes, the homozygous hemoglobinopathies, and the group of variants that do not cause disease will be discussed in the next several pages; some recent reviews (B36, C5, C24, H26, H27, H33, H34, H37, H52, L12, M16, P14) should also be consulted.

3.1.1. *Variants with Substitutions of Residues Participating in Contacts with Heme Groups*

These variants usually cause a congenital nonspherocytic hemolytic anemia in the heterozygote. The substitutions concern replacements of nonpolar contacts with heme which are essential for maintaining the hemoglobin molecule functional. Even small stereochemical changes apparently result in a considerable instability of the molecule. The disease in these patients varies from mild to severe with hemolysis of the red cells, hypochromia, low MCHC, and reticulocytosis. Characteristic is the presence of inclusion bodies (Heinz bodies) which are easily demonstrated by supravital staining. Incubation of the cells for 24 hours without glucose usually facilitates the formation of these bodies. The precipitate is mainly denatured hemoglobin. Some heterozygotes excrete dipyrroles of the bilifuscin-mesobilifuscin type which gives the urine a characteristic dark color. There is considerable evidence that loss of heme from the mutant chain might frequently underlie the denaturation of the variant into Heinz bodies (H15, J5, R1) although many variants precipitate with the heme group still attached. The denatured hemoglobin molecules are probably tightly bound to the red cell membrane probably involving the membrane thiols in the attachment (J4, J8). Red cells with Heinz bodies are easily trapped in the spleen, and their destruction will follow. The fixation of the Heinz bodies to the cell membrane also results in a change in osmotic and mechanical fragilities; the life-span of the cells is shortened considerably. Figure 11 summarizes the pathogenesis of the Heinz body hemolytic anemia.

Table 2 lists the residues in the α and the β chains that are in contact with heme and the variants that have been discovered. Many of these variants are familial; examples are the hemoglobins Köln, Zurich, Genova, Richmond, Louisville. Others, as Hb-Hammersmith and Hb-

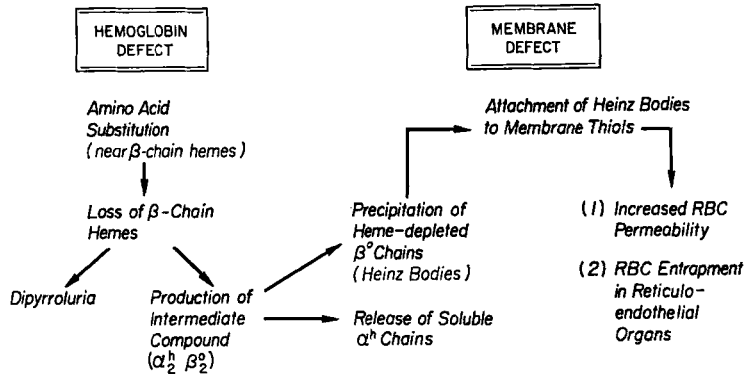

FIG. 11. Scheme of the pathogenesis of congenital Heinz body hemolytic anemia. From (J4), H. S. Jacob, *Seminars in Hematology* **7**, 341 (1970), with permission of the author and publisher.

Sabine, represent spontaneous mutations. The effect of the substitution can be extremely mild (Hb-Richmond, Hb-I-Toulouse) to severe (Hb-Bibba, Hb-Hammersmith). Subjects with Hb-Zurich are asymptomatic unless exposed to oxidant drugs causing an acute hemolytic episode with formation of Heinz bodies. Particularly severe is the lesion in Hb-Hammersmith because the removal of phenylalanine CD1 is accompanied by the introduction of the polar OH group of serine which probably facilitates the entry of water into the heme pocket which causes the heme group to drop out (P14). Substitution of this phenylalanine by leucine, as in Hb-Louisville, results in a much less severe anemia (K9).

Three variants, the β chain abnormalities Hb-Köln, Hb-Kansas, and Hb-Richmond, have substitutions of residues that also participate in the $\alpha_1\beta_2$ contact.

Replacement of the proximal histidyl residue (87 in the α chain and 92 in the β chain), which occupies the fifth coordinate position of the iron of heme, by a tyrosyl residue (Hb-M-Iwate and Hb-M-Hyde Park, respectively) results in the formation of Met(=ferri) hemoglobin. It seems likely that the iron atoms in these two hemoglobins are covalently linked to the distal histidyl residue (α-58 and β-63, respectively) whereas an ionic link is present between the iron atom and the OH group of the tyrosyl residue in position α-87 or β-92. Replacement of the distal histidyl residue, α-58 or β-63, by a tyrosyl residue is found in the methemoglobins M-Boston and M-Saskatoon. This substitution also results in an ionic link between the phenolic oxygen of this residue and the ferric iron. The fifth methemoglobin, Hb-M-Milwaukee, has similar properties be-

TABLE 2

HEMOGLOBIN VARIANTS WITH SUBSTITUTIONS OF A RESIDUE PARTICIPATING IN THE CONTACT WITH THE HEME GROUP

Position[a]	Residue		Substitution	Name	Comments	References
			The α chain			
32-B13	Met					
39-C4	Thr					
42-C7	Tyr					
43-CD1	Phe	→	Val	Torino	Inclusion bodies; ↓O_2 affinity; unstable	(B29)
45-CD3	His					
46-CD4	Phe					
58-E7	His	→	Tyr	M-Boston	Methemoglobin; cyanosis	(G8)
62-E11	Val					
83-F4	Leu					
86-F7	Leu					
87-F8	His	→	Tyr	M-Iwate	Methemoglobin; cyanosis	(J10)
91-FG3	Leu					
93-FG5	Val					
97-G4	Asn					
98-G5	Phe					
101-G8	Leu					
129-H12	Leu					
132-H15	Val					
136-H19	Leu	→	Pro	Bibba	Inclusion bodies; ↑dissociation; unstable	(K18, S53)
			The β chain			
31-B13	Leu					
38-C4	Thr					
41-C7	Phe					
42-CD1	Phe	→	Ser	Hammersmith	Inclusion bodies; cyanosis; unstable; ↓oxygen affinity	(D1)
		→	Leu	Louisville	Inclusion bodies; mild cyanosis; unstable; ↓oxygen affinity	(K9)
44-CD3	Ser					
45-CD4	Phe					
63-E7	His	→	Tyr	M-Saskatoon	Methemoglobin; cyanosis	(H4)
		→	Arg	Zurich	Hemolytic anemia after sulfa drugs; ↑O_2 affinity	(F12, M34)

(*Continued*)

TABLE 2 (*Continued*)

Position[a]	Residue		Substitution	Name	Comments	References
66-E10	Lys	→	Glu	Toulouse	Mild anemia; methemoglobin	(R28)
67-E11	Val	→	Glu	M-Milwaukee	Methemoglobin; cyanosis	(P19, U2)
		→	Ala	Sydney	Hemolytic anemia; inclusion bodies; unstable	(C4)
		→	Asp	Bristol	Hemolytic anemia; methemoglobin; $\downarrow O_2$ affinity	(H26)
70-E14	Ala					
71-E15	Phe	→	Ser	Christchurch	Hemolytic anemia; inclusion bodies; unstable	(C6)
88-F4	Leu	→	Pro	Santa Ana	Hemolytic anemia; inclusion bodies; β chain no heme	(H16)
		→	Arg	Borås	Hemolytic anemia; methemoglobin; $\uparrow O_2$ affinity	(H17)
91-F7	Leu	→	Pro	Sabine	Hemolytic anemia; unstable low heme binding	(S11)
92-F8	His	→	Tyr	M-Hyde Park	Methemoglobin; cyanosis	(S40)
96-FG3	Leu					
98-FG5	Val	→	Met[b]	Köln	Hemolytic anemia; unstable; $\uparrow O_2$ affinity	(C3)
102-G4	Asn	→	Thr[b]	Kansas	Cyanosis; unstable; $\downarrow O_2$ affinity	(B48)
		→	Lys[b]	Richmond	↑Dissociation; asymmetric hybrids	(E3)
103-G5	Phe					
106-G8	Leu					
137-H15	Val					
141-H19	Leu	→	Arg	Olmstead	Hemolytic anemia; unstable	(F1, L32)

[a] Position is given by the residue number in the chain and by that in the helix or intrahelical segment.

[b] Position participates also in $\alpha_1\beta_2$ contact.

cause of the interaction of the glutamyl residue in position 67 with the iron of the heme group. Substitution of histidyl residue in position 63 by an arginyl residue, as in Hb-Zurich, does not interfere with the reversible combination of iron with oxygen.

The loss of a vital contact with heme may also result in loss of heme (Hb-Santa Ana, Hb-Hammersmith, Hb-Sydney) and in changes in oxygen carrying properties. When a prolyl residue is introduced (Hb-Bibba, Hb-Santa Ana, Hb-Sabine) disruption of the conformation of the helix will probably add to the magnitude of the effects.

3.1.2. *Variants with Substitutions in the $\alpha_1\beta_1$ Contact*

Table 3A lists the residues of the α and the β chain which are involved in the $\alpha_1\beta_1$ contact; the positions of these residues within the chains can also be found in Figs. 1 and 2. Six variants involving substitutions in this contact have been reported (Table 3A). Three (Hb-G-Chinese, Hb-Chiapas, and Hb-Khartoum) do not cause clinical symptoms in the heterozygote. Carriers of the other three variants suffer from a mild hemolytic anemia with erythrocytosis and an increased oxygen affinity in the Hb-Tacoma heterozygote, and a decreased oxygen affinity in the carrier of the Yoshizuka variant.

3.1.3. *Variants with Substitutions in the $\alpha_1\beta_2$ Contact*

Table 3B lists the ten amino acid residues in the α chain and the nine amino acid residues in the β chain which form the area of lesser contact between unlike chains, the $\alpha_1\beta_2$ contact. As pointed out before, this contact undergoes the greatest shift during the oxygenation and deoxygenation process.

Substitutions of amino acid residues in two positions of this contact, α-92-Arg and α-95-Pro, have been observed in five variants. Four of these variants (Hb-St. Luke's has not been analyzed) have an increased affinity for molecular oxygen which is particularly evident for the variants Hb-Chesapeake and Hb-J-Capetown. Erythrocytosis is a common feature in carriers of these abnormalities. The oxy derivatives of the hemoglobins G-Georgia and Rampa are completely dissociated into dimers, and association into tetramers is observed when the oxygen is removed (S53). Such a phenomenon is not as evident for the Hb-St. Luke's variant (H38). Reduction in the heme–heme interaction, a common finding in these variants, can be explained by an increased dissociation of the oxy derivatives.

Six of the eight β chain variants with substitutions in the $\alpha_1\beta_2$ contact exhibit an increased oxygen affinity. This results in clinical symptoms, especially erythrocytosis, which can be correlated with the increased demand for hemoglobin to compensate for the decreased release of oxygen to the tissues. The dissociation of the tetrameric molecule into asymmetric dimers is often altered in these cases, as has been demonstrated for the hemoglobins Kansas and Richmond. The Hb-Kansas variant is

TABLE 3A

HEMOGLOBIN VARIANTS WITH SUBSTITUTIONS OF A RESIDUE PARTICIPATING IN THE $\alpha_1\beta_1$ CONTACT

Position[a]	Residue		Substitution	Name	Comments	References
				The α chain		
30-B11	Glu	→	Gln	G-Chinese	No abnormal properties	(S67)
31-B12	Arg					
34-B15	Leu					
35-B16	Ser					
36-C1	Phe					
103-G10	His					
104-G11	Cys					
106-G13	Leu					
107-G14	Val					
111-G18	Ala					
114-GH2	Pro	→	Arg	Chiapas	No abnormal properties	(J18)
117-GH5	Phe					
119-H2	Pro					
122-H5	His					
123-H6	Ala					
126-H9	Asp					
				The β chain		
30-B12	Arg	→	Ser	Tacoma	↑O_2 affinity; erythrocytosis; unstable	(B72)
33-B15	Val					
34-B16	Val					
35-C1	Tyr	→	Phe	Philly	Mild hemolytic anemia; unstable	(R20)
51-D2	Pro					
55-D6	Met					
108-G10	Asn	→	Asp	Yoshizuka	Mild hemolytic anemia; ↓O_2 affinity	(I1)
112-G14	Cys					
115-G17	Ala					
116-G18	His					
119-GH2	Gly					
122-GH5	Phe					
123-H1	Thr					
124-H2	Pro	→	Arg	Khartoum	No abnormal properties	(C15)
125-H3	Pro					
127-H5	Glu					
128-H6	Ala					
131-H9	Glu					

[a] See footnote *a* of Table 2.

TABLE 3B

HEMOGLOBIN VARIANTS WITH SUBSTITUTIONS OF A RESIDUE PARTICIPATING IN THE $\alpha_1\beta_2$ CONTACT

Position[a]	Residue		Substitution	Name	Comments	References
			The α chain			
38-C3	Thr					
41-C6	Thr					
42-C7	Tyr					
91-FG3	Leu					
92-FG4	Arg	→	Leu	Chesapeake	Erythrocytosis; ↑O_2 affinity	(C12, C18)
		→	Gln	J-Capetown	Mild erythrocytosis; ↑O_2 affinity	(B56)
93-FG5	Val					
94-G1	Asp					
95-G2	Pro	→	Leu	G-Georgia	Oxy derivative; dissociation in dimers; ↑O_2 affinity	(H51, S54)
		→	Ser	Rampa	Oxy derivative; dissociation in dimers; ↑O_2 affinity	(J24, S54)
		→	Arg	St. Luke's	Much less dissociated	(H38)
96-G3	Val					
140-H23	Tyr					
			The β chain			
36-C2	Pro					
37-C3	Trp	→	Ser	Hirose	↑O_2 affinity	(Y1)
39-C5	Gln					
40-C6	Arg					
97-FG4	His	→	Glu	Malmö	↑O_2 affinity; erythrocythemia	(L32)
98-FG5	Val	→	Met[b]	Köln	Hemolytic anemia; unstable; ↑O_2 affinity	(C3)
99-G1	Asp	→	His	Yakima	↑O_2 affinity; erythrocytosis	(J16, N14)
		→	Asn	Kempsey	↑O_2 affinity; erythrocytosis	(R14)
		→	Tyr	Ypsilanti	↑O_2 affinity; erythrocytosis; asymmetric hybrids	(G10)
101-G3	Glu					
102-G4	Asn	→	Thr[b]	Kansas	Cyanosis; unstable; ↓O_2 affinity	(B48)
		→	Lys[b]	Richmond	Dissociation; asymmetric hybrids	(E3)

[a,b] See footnotes *a* and *b* of Table 2.

TABLE 4

PATHOLOGICAL HEMOGLOBIN VARIANTS WITH SUBSTITUTIONS OF RESIDUES IN POSITIONS NOT INVOLVED IN CONTACTS WITH HEME OR BETWEEN CHAINS

Position[a]	Residue	Substitution	Name	Comments	References
			The α chain		
16-A14	Lys →	Glu	I-Philadelphia	Target cells; increased alkali resistance	(S7)
27-B8	Glu →	Gly	Ft. Worth	Microcytosis; hypochromia	(S12)
47-CE5	Asp →	His	Sinai	Unstable	(C13, O4)
47-CE5	Asp →	Glu	L-Ferrara		(N1)
50-CE8	His →	Asp	J-Sardegna	Anemia	(T2)
80-F1	Leu →	Arg	Ann Arbor	Unstable	(R37)
84-F5	Ser →	Arg	Etobicoke	Unstable; ↑O_2 affinity	(C31)
90-FG2	Lys →	Asn	Broussais	Anemia	(V5)
102-G9	Ser →	Arg	Manitoba	Slightly unstable	(C32)
112-G19	His →	Glu	Dakar	Mild hemolytic anemia	(R27)
			The β chain		
2-NA2	His →	Tyr	Tokuchi	Mild anemia	(S37)
6-A3	Glu →	Val	Hb-S	Sickling	(H61)
6-A3	Glu →	Lys	Hb-C	Crystallization in RBC; mild hemolytic anemia in homozygote	(H61)
9-A6	Ser →	Cys	Porto Alegre	Polymerization	(B47)
14-A12	Leu →	Arg	Sogn	Slightly unstable	(M25)
24-B6	Gly →	Arg	Riverdale-Bronx	Hemolytic anemia; unstable	(R11)
24-B6	Gly →	Val	Savannah	Hemolytic anemia; unstable	(H55)
26-B8	Glu →	Lys	Hb-E	Mild hemolytic anemia in homozygote; ↓O_2 affinity	(H63)
28-B10	Leu →	Pro	Genova	Hemolytic anemia; unstable	(S2)
32-B14	Leu →	Pro	Perth	Unstable	(H27)
74-E18	Gly →	Asp	Shepherds Bush	Mild hemolytic anemia; ↑O_2 affinity	(W23)
76-E20	Ala →	Glu	Seattle	Mild anemia; ↓O_2 affinity	(S60)
90-F6	Glu →	Lys	Agenogi	↓O_2 affinity	(M19)
93-F9	Cys →	blocked	Ube I	Unstable; hemolytic anemia	(S38)
108-G10	Asn →	Asp	Yoshizuka	Mild anemia	(I1)
111-G13	Val →	Phe	Peterborough	Mild anemia; ↓O_2 affinity	(M27)
117-G19	His →	Arg	Hb-P	Hypochromic anemia	(S10)

TABLE 4 (*Continued*)

Position[a]	Residue	Substitution	Name	Comments	References
130-H8	Tyr →	Asp	Wien	Mild hemolytic anemia	(J19)
136-H14	Gly →	Asp	Hope	Unstable	(M18)
145-H23	Tyr →	Cys	Rainier	Polycythemia; ↑O_2 affinity; resistance to alkali	(G15, H5)
145-H23	Tyr →	His	Bethesda	Polycythemia; no resistance to alkali	(H5)
146-H24	His →	Asp	Hiroshima	Polycythemia; O_2 affinity	(P18)

[a] See footnote *a* of Table 2.

of particular interest because it has a greatly decreased affinity for oxygen and causes cyanosis in the carrier. Hb-Richmond, on the contrary, does not produce ill effects.

Hb-Richmond and Hb-Ypsilanti form hybrids species of the type $\alpha_2\beta^A\beta^X$ (β^X refers to the abnormal β chain) which are stable at low temperature. When Hb-Richmond is mixed with an α chain variant, such as Hb-I, a hemoglobin tetramer with four different polypeptide chains, $\alpha^A\alpha^I\beta^A\beta^R$, is formed. This observation together with data describing the dissociation properties of the α chain variants Hb-G-Georgia and Hb-Rampa provide further evidence that the $\alpha_1\beta_2$ contact is the contact that is broken during dissociation of the tetrameric hemoglobin molecule.

3.1.4. *Other Pathological Variants*

Table 4 lists ten α chain variants and 22 β chain variants which may cause (very) mild to more severe clinical symptoms.

Four of the α chain variants listed are less stable upon heating. One of these, Hb-Manitoba, has the only known substitution in the central cavity of the hemoglobin molecule. Although this variant is slightly unstable, no disease is present in affected individuals.

The hemoglobins S and C are the best known β chain variants and will be discussed in a later paragraph.

Hb-Porto Alegre (β 9 Ser → Cys) and Hb-Rainier (β 145 Tyr → Cys) are variants in which an additional cysteinyl residue is introduced. This may cause polymerization through the formation of —S—S— bridges and larger molecules have indeed been observed for Hb-Porto Alegre. Hb-Rainier has an increased affinity for molecular oxygen resulting in erythrocytosis in the affected individual. The disulfide bonding apparently also causes an increased resistance of the variant against alkali.

Hb-E shows a mild instability that is not due to the direct effect of the Glu → Lys substitution in position 26 because this residue does not participate in any known contact. However, the introduction of a lysyl residue in this position eliminates the normal neutralization of the charge on the arginyl residue in position 30 and disrupts a system of hydrogen bonds between Glu 26-B8 and His 116-G18 and His 117-G19.

Several variants with substitutions of residues not participating in the contact with heme are unstable, and a mild to severe hemolytic anemia with Heinz body formation is present in heterozygotes. Examples are Hb-Riverdale-Bronx, Hb-Savannah, Hb-Genova, Hb-Perth, Hb-Shepherds Bush, Hb-Seattle, Hb-Wien, and others. Two of these, Hb-Genova and Hb-Perth, concern substitutions of a leucyl residue in a helical position by a prolyl residue which apparently disrupts the conformation of the helix sufficiently to alter the properties of this variant considerably. The substitution of the glycyl residue 24-B6 by arginyl (Hb-Riverdale-Bronx) or by valyl (Hb-Savannah) concerns a residue that is in close spatial contact with glycyl residue 64-E8 (see Fig. 2). Substitution of this residue by a larger residue changes the tertiary structure in such a way that indirectly the $\alpha_1\beta_2$ contact is affected as well as the relationship of the heme group to the polypeptide chain because of a considerable distortion of the E-helix where the distal heme-linked histidyl residue is adjacent to the Gly 64-E8 residue and four additional heme linked contacts are also present.

Other substitutions on the inside of a subunit which give rise to disease are found in the variants Hb-Ann Arbor (α 80), Hb-Sogn (β 14), and Hb-Wien (β 130). In these variants a polar residue (Arg or Asp) replaces a leucyl or a tyrosyl residue in a nonpolar region. Apparently such a polar residue cannot be accommodated in the hydrophobic interior, and instability of the entire molecule results when the residue tries to reach the exterior of the molecule or to interact with another side chain in its vicinity (H27).

3.1.5. *Variants without Clinical Abnormalities*

Table 5 lists 24 α chain variants and 35 β chain variants. Nearly all substitutions are at the surface of the molecule. None causes ill effects in heterozygotes, but some may give mild disease in homozygous state.

3.1.6. *Sickle Cell Anemia, Hb-C Disease, and Related Abnormalities*

The β chain variant, Hb-S or $\alpha_2\beta_2{}^{6Val}$, is essentially innocuous in the heterozygote, but the homozygote is usually severely affected. The Hb_{β^S} gene is widely distributed and its incidence probably exceeds that of all other variants combined. Sickle cell disease causes some 60,000–80,000

TABLE 5
HEMOGLOBIN VARIANTS WITHOUT CLINICAL IMPORTANCE

Position[a]	Residue		Substitution	Name	References
			The α chain		
5-A3	Ala	→	Asp	J-Toronto	(C30)
12-A10	Ala	→	Asp	J-Paris	(R26)
15-A13	Gly	→	Asp	J-Oxford	(L17)
22-B3	Gly	→	Asp	J-Medellin	(G13)
23-B4	Glu	→	Gln	Memphis	(K25)
23-B4	Glu	→	Lys	Chad	(B61)
23-B4	Glu	→	Val	G-Audhali	(M5)
51-CE9	Gly	→	Arg	Russ	(R16)
54-E3	Gln	→	Arg	Shimonoseki	(H3)
54-E3	Gln	→	Glu	J	(L1)
57-E6	Gly	→	Arg	L-Persian Gulf	(R2)
57-E6	Gly	→	Asp	J-Norfolk	(B2)
60-E9	Lys	→	Asn	Zambia	(B16)
68-E17	Asn	→	Asp	Ube-2	(M20)
68-E17	Asn	→	Lys	G-Philadelphia	(B5)
74-EF3	Asp	→	His	Taichung	(L31)
75-EF4	Asp	→	His	Q	(L31)
78-EF7	Asn	→	Lys	Stanleyville-II	(V1)
85-F6	Asp	→	Asn	G-Norfolk	(J19)
85-F6	Asp	→	Tyr	Atago	(F13)
112-G19	His	→	Asp	Hopkins-II	(S52)
115-GH3	Ala	→	Asp	J-Tongariki	(A1, G4)
116-GH4	Glu	→	Lys	O-Indonesia	(B6)
141	Arg	→	Pro	Singapore	(C15)
			The β chain		
6-A3	Glu	→	Ala	G-Makassar	(B42)
7-A4	Glu	→	Gly	G-San José	(H14)
7-A4	Glu	→	Lys	G-Siriraj	(T12)
16-A13	Gly	→	Asp	J-Baltimore	(B7)
16-A13	Gly	→	Arg	D-Bushman	(W1)
17-A14	Lys	→	Glu	Nagasaki	(M2)
22-B4	Glu	→	Lys	E-Saskatoon	(V3)
22-B4	Glu	→	Ala	Hsin-Chu	(B38)
22-B4	Glu	→	Gly	G-Taipei	(B41)
25-B7	Gly	→	Arg	G-Taiwan-Ami	(B44)
43-CD2	Glu	→	Ala	G-Galveston	(B57)
46-CD5	Gly	→	Glu	K-Ibadan	(A10)
47-CD6	Asp	→	Asn	G-Copenhagen	(S42)
50-D1	Thr	→	Lys	Edmonton	(L4)
56-D7	Gly	→	Asp	J-Bangkok	(C18)
58-E2	Pro	→	Arg	Dhofar	(M6)
61-E5	Lys	→	Glu	N-Seattle	(J17)
61-E5	Lys	→	Asn	Hikari	(S39)

(*Continued*)

TABLE 5 (*Continued*)

Position[a]	Residue	Substitution	Name	References
69-E13	Gly	→ Asp	J-Cambridge	(S42)
73-E17	Asp	→ Asn	Korle Bu	(K22)
77-EF1	His	→ Asp	J-Iran	(R4)
79-EF3	Asp	→ Asn	G-Accra	(L9)
80-EF4	Asn	→ Lys	G-Szuhu	(B39)
87-F3	Thr	→ Lys	D-Ibadan	(W7)
94-FG1	Asp	→ Asn	Oak Ridge	(S16)
95-FG2	Lys	→ Glu	N-Baltimore	(C17)
113-G15	Val	→ Glu	New York	(R10)
120-GH3	Lys	→ Glu	Hijiyama	(M21)
121-GH4	Glu	→ Gln	D-Los Angeles	(B1)
121-GH4	Glu	→ Lys	O-Arab	(B6)
126-H4	Val	→ Glu	Hofu	(M22)
129-H7	Ala	→ Asp	J-Taichung	(B40)
129-H7	Ala	→ —	K-Cameroon	(A10)
132-H10	Lys	→ Gln	K-Woolwich	(A10)
136-H14	Gly	→ Asp	Hope	(M18)
143-H21	His	→ Glu or Asp	Kenwood	(B20)

[a] See footnote *a* of Table 2.

deaths each year in African children and incapacitates many. Homozygous Hb-S disease occurs in about 1:500 Negro newborns. The pathology of the disease is extensively reviewed by Song (S56).

The unique chemical characteristic is the replacement of Glu 6-A3 by a valyl residue resulting in a decrease in the solubility of the deoxygenated form. Thus, Hb-S molecules in concentrated solution will, upon deoxygenation, aggregate and consequently deform the red cell because of distortion of the cellular architecture. There is some question about the exact mechanism involved in the formation of intermolecular bonds. The hypothesis based on observations by Murayama (M35) suggests the formation of a hydrophobic bond between Val 1-NA1 and Val 6-A3 which results in cyclization by hydrogen bonding between Val 1-NA1 and Thr 4-A1. This cyclic structure is located at the surface of the molecule and should fit into a complementary part of an α chain of a second hemoglobin molecule, thus allowing stacking of the molecules. This hypothesis has been rather enthusiastically accepted. However, X-ray crystallographic studies of deoxyhemoglobin fail to suggest a location for or possible identity of the complementary part which should serve as a lock for the key offered by the amino terminal ring structure of the β^S chain. Of interest is also the observation that the interaction between 2,3-DPG (which likely binds to deoxyhemoglobin at residues

Val 1-NA1, Lys 82-EF6, and His 143-H21) and deoxy Hb-S does not produce configuration changes which influence the aggregation of the molecules (B35). Moreover, cyanate (HN=C=O) which carbamylates amino terminal residues of proteins (R—NH—CO—NH_2) is an effective inhibitor of sickling of erythrocytes *in vitro* and of gelling of concentrated solutions of deoxyhemoglobin S (C9). Addition of some specific hemoglobin variants, Hb-O-Arab or $\alpha_2\beta_2^{121\ Lys}$ (M16) and Hb-Korle Bu or $\alpha_2\beta_2^{73\ Asn}$ (B53), to deoxy Hb-S seems to influence the aggregation of polymers. The observation that a similar effect has been observed for Hb-C-Harlem, which is a variant with two amino acid substitutions in each β chain, namely $\beta^{6\ Glu\rightarrow Val}$ as in β^S and $\beta^{73\ Asp\rightarrow Asn}$ as in Hb-Korle-Bu, might be of significance. These data suggest that the introduction of an asparagine residue in position 73 of the β^S chain alters some site of interaction other than the site determined by the $\beta^{6\ Val}$ substitution. Studies like these might ultimately suggest a more definite mechanism for the sickling phenomenon. But, the elucidation of this mechanism awaits the determination of the three-dimensional structure of deoxy-Hb-S by X-ray crystallography.

Recently, treatment of the crisis phase of sickle cell anemia by administration of large amounts of urea in a solution of invert sugar has been introduced (N2). The rationale of this therapy is the presumed disruption of the hydrophobic bonds between the Hb-S molecules. The use of this well-known protein denaturant in patients with this disorder has created considerable controversy (the reader is referred to the many letters to the editors of, for instance, the *New England Journal of Medicine,* 1970 and 1971). In view of these differences of opinion and of the incertitude as to the mechanism of the sickling phenomenon the statement by Nalbandian *et al.* (N2) that "contrary to general opinion, sickle cell anemia is now among the best understood of diseases. There is now a molecular basis for the pathogenesis, . . . , and the treatment of this lethal genetic affliction" is imprudent and unfortunate.

There are four additional variants with substitutions of either Glu 6-A3 or Glu 7-A4 (Tables 4 and 5); these variants are Hb-C (β 6-A3 Lys) and Hb-G-Makassar (β 6-A3 Ala), Hb-G-San José (β 7-A4 Gly), and Hb-C-Siriraj (β 7-A4 Lys). None of these abnormalities causes a sickling phenomenon comparable to that found for red cells with Hb-S. Hb-C has a slightly decreased solubility (H39) which probably explains the frequent occurrence of intracellular crystals of Hb-C in blood smears of Hb-C homozygotes.

Hb-S (and Hb-C) is often inherited with some other β chain variant or with β-thalassemia. For a discussion of biochemical and pathological aspects of SC disease, SD disease, S-β-thalassemia, the reader is re-

ferred, for example, to the many review articles listed on page 1 and to reference S56. Certain aspects of the interaction of Hb-S and of other β chain abnormal variants with β-thalassemia are discussed in Section 4.

3.1.7. *Variants with Two Amino Acid Substitutions*

One variant with two amino acid substitutions, Hb-C-Harlem, has been discovered (B50, B51); the two substitutions are present in the β-polypeptide chain (Table 6). A second possible variant, Hb-C-Georgetown (B49), has not been identified, and the possibility that this abnormality is identical to Hb-C-Harlem cannot be excluded. It has been suggested that the Hb-$_{\beta^{C\text{-}Harlem}}$ gene originated when a second mutation occurred in an individual carrying the Hb$_{\beta^S}$ gene (the β chain of Hb-S has the same abnormality as one of the two amino acid substitutions in the β chain of Hb-C-Harlem) or by homologous crossing-over within the β chain loci between the β-6 and β-73 determinants.

3.1.8. *Variants with Deletions*

Crossing-over or recombination at homologous loci is a normal process and results in a redistribution of parental genes. However, mispairing may lead to the formation of abnormal structural genes whose

TABLE 6
SPECIAL HEMOGLOBIN VARIANTS

Variants			References
A. *Hemoglobin with two substitutions in β chain*			
C-Harlem	6 (A3)[a]	Glu → Val	(B50, B51)
	73 (E17)	Asp → Asn	
B. *Hemoglobins with deletions*			
Hb-Leiden	Residue 6 or 7 (= Glu) deleted		(J22)
Hb-Freiburg	Residue 23 (= Val) deleted		(J14)
Hb-Tochigi	Residues 56 through 59 deleted		(S41)
Hb-Gun Hill	Residues 93 through 97 deleted		(B64, R19)
C. *The Lepore hemoglobins*			
Hb-Lepore-Washington	δβ Crossover between Gln 87δ and Arg 116β		(B3, B9, L2)
Hb-Lepore-Hollandia	δβ Crossover between Ala 22δ and Thr 50β		(B19, N10)
Hb-Lepore-Baltimore	δβ Crossover between Ser 50δ and Ala 86β		(O5)
Hb-Miyada	βδ Crossover between Thr 12β and Ala 22δ		(O1, U1)

[a] Refers to position in chain and to that in helix, respectively.

product can readily be identified. Examples are: (a) nonhomologous, unequal crossing over leading to *gene duplication* and *gene deletion;* (b) homologous, unequal crossing over leading to the formation of *fusion genes;* (c) abnormal pairing between two homologous genes may result into *internal duplication genes* and *internal deletion genes.*

Four β chain variants have been described in which deletions of amino acid residues are present (Table 6); these abnormal β chains are the product of a structural β chain gene with loss of one or more than one base triplet (internal deletion gene).

Sometimes deletions of amino acid residues may have a profound influence on the physicochemical and functional properties of the variant. The deletion of valyl residue in position β 23 (B5), for instance, results in considerable changes in spectral absorption, in affinity for molecular oxygen, and in heat stability. The deletion of the five residues in Hb-Gun Hill is near the heme position, and heme will not bind to the altered β chain. However, when the deletion occurs near a terminus, as in Hb-Leiden, the effect is minimal.

3.1.9. *Variants with δ-β or β-δ Chains*

Three hemoglobin variants have been described which have been named the Lepore variants. The first was described in 1958 (G7) in association with clinical and hematological features of thalassemia. This variant, Hb-Lepore-Washington, is present in different populations in Italy (S46), Rumania (R34), Yugoslavia (D12), Greece, where it is known as Hb-Pylos (F6), Cyprus (B23), and others. Homozygous subjects show a complete absence of the hemoglobins A and A_2, a finding which suggests that the abnormality affects the Hb_β and Hb_δ loci. Structural analyses (B3, B9, L2) have shown a sequence of the non-α chain which is similar to the δ chain from residues 1 to 87 and to that of the β chain from residues 116 to 146. The δ-β chain of Hb-Lepore is 146 residues long. Thus, the non-α chain of Hb-Lepore-Washington is the product of a $Hb_{\delta-\beta}$ structural gene which resulted from an unequal crossing-over between the Hb_δ and Hb_β structural loci. Such a crossing-over will give a daughter chromosome which carries a $Hb_{\delta-\beta}$ locus without the original Hb_δ and Hb_β loci (deletion chromosome), and a second daughter chromosome which carries a $Hb_{\beta-\delta}$ fusion gene plus the original Hb_δ and Hb_β loci (duplication chromosome). The absence of Hb-A and Hb-A_2 in Hb-Lepore homozygotes and data from structural analyses have indicated that indeed the presence of a $Hb_{\beta-\delta}$ fusion gene with deletion of Hb_β and Hb_δ structural genes will explain the Hb-Lepore abnormality satisfactorily.

Two additional Lepore hemoglobins, Hb-Lepore-Hollandia and Hb-

Lepore-Baltimore, have been discovered. Both show a comparable abnormality, but the crossovers between the Hb_δ and Hb_β structural loci apparently occurred at different locations, namely between nucleotides coding for $\delta^{22\ Ala}$ and $\beta^{50\ Thr}$, and between those coding for $\delta^{50\ Ser}$ and $\beta^{86\ Ala}$, respectively (Table 6). Hb-Lepore-Hollandia is found on New Guinea, and Hb-Lepore-Baltimore was discovered in a person of Afro-American descent.

The recent discovery of Hb-Miyada is of interest because structural analysis of the non-α chain of this variant shows that this polypeptide chain is the product of a fusion gene which results from a crossover between nucleotides coding for $\beta^{12\ Thr}$ and $\delta^{22\ Ala}$ (Table 6). Thus, this β–δ chain is synthesized by a $Hb_{\beta\text{-}\delta}$ fusion gene which is present on a duplication chromosome. The additional presence of the original Hb_δ and Hb_β structural genes is indicated because the Hb-Miyada heterozygote is clinically well, and apparently is able to synthesize normal quantities of β and δ chains.

3.1.10. *Variants with Elongated Chains*

Recently two hemoglobin variants with elongated chains have been found (E4, F10, M17). Hb-Constant Spring is an unusual α chain variant which is primarily present in individuals of Cantonese extraction. It constitutes 0.1–0.3% of the total hemoglobin in heterozygotes but is synthesized in considerably larger amounts (some 4–4.5%) when inherited together with an α-thalassemia gene (E4, M17). The abnormal α chain might well be 31 residues longer than the normal α chain; the extra residues are attached to the carboxy-terminal end (M17). Two extra peptides have been found in tryptic digests of this α chain which have the sequences Trp-Ala-Ser-Glu-Arg and Ala-Leu-Leu-Pro-Ser-Leu-His-Arg (E4). The genetic event leading to the formation of this unusual α chain is likely a mutation of the terminating codon (UAA or UAG) in the normal Hb_α structural gene, or perhaps in a minor Hb_α structural gene. The possibility that this α chain is a crossover product can also not be excluded. The low production of the abnormality in heterozygotes who are clinically healthy, and the 10-fold increase when an α thalassemia determinant is present *in trans* seem to support the suggestion that more than one Hb_α structural locus is present on one chromosome.[1]

[1] NOTE ADDED IN PROOF: Recently Clegg, Weatherall, and Milner (1970) [*Nature* **234**, 337–340] have given the sequence of the 31 amino acid residues, namely,

140 150
Tyr-Arg-Gln-Ala-Gly-Ala-Ser-Val-Ala-Val-Pro-Pro-Ala-Arg-Trp-Ala-Ser-Gln-
160 170
Arg-Ala-Leu-Leu-Pro(His,Ser,Leu)-Arg-Pro-Phe-Leu-Val-Phe-Glu.

The second abnormality, Hb-Tak, is a β chain variant with an electrophoretic mobility slightly faster than that of Hb-S. Hb-Tak amounts to about 40% of the total hemoglobin and has been found in two individuals from Northwestern Thailand. The β chain is extended at the carboxy-terminal end by ten amino acid residues, which are in the following tentative sequence (F10):

145–146–147–148–149–150–151–152–153–154–155–156
Tyr–His–Thr–Lys–Leu–Leu–Ala (Ser, Leu, Asn)Phe–Tyr

It is of great interest that the Hb-Tak chain is produced in amounts similar to that of most β chain variants. The presence of a threonyl residue in position 147 is unexpected if one assumes that the formation of this β chain is due to a mutation of a terminating codon UAA or UAG. The study of this hemoglobin might well be of importance for a better insight into the mechanism of normal chain termination and in the nature of the DNA between two genes. The presence of Hb-Tak is not associated with any major hematological disorder.

3.1.11. *Relative Rates of Synthesis of Variant Chains in Heterozygotes*

Most individuals who are heterozygous for a variant β chain have less abnormal hemoglobin than normal Hb-A. The presence of only 30–40% of abnormal β chain is probably due to a decreased rate of synthesis. This has been demonstrated for the β^S chain (B15, B60, H12) and the β chain of Hb-Riverdale-Bronx (B15). It may be that the production of messenger RNA for the abnormal β chain is decreased or that the translation of the abnormal messenger RNA occurs at a reduced rate. It is of interest that the synthesis in reticulocytes of the heme-free β chain of Hb-Gun Hill is considerably larger than that of the β^A chain suggesting that the heme-globin binding might influence the rate of production of β polypeptide chains (R18).

The relative amount of β chain variants (and of Hb-A_2) is markedly decreased in individuals who also have a megoloblastic anemia or an iron deficiency anemia (H8, L15). Acquired conditions apparently will influence the activity of specific genomes.

The synthesis of α-chain variants might be similarly affected. However, the relative amounts of these variants in heterozygotes differ considerably namely from 5% for Hb-Ft. Worth (α 27 Glu $\rightarrow$ Gly) to some 35–40% for many other α chain variants. Recent studies have indicated that indeed the abnormal α chain is synthesized at a diminished rate compared to the α^A chain in both reticulocytes and nucleated red cells of patients heterozygous for the α chain variant I-Philadelphia or α 16-A14 (Lys $\rightarrow$ Glu) (E6). Another possible explanation for a low quantity of α chain variants in heterozygotes is based on the assump-

tion that a duplicated Hb_α structural locus may be present; thus, the variant would be the product of an allele of only one of the two Hb_α genes and therefore be produced in a relatively low amount. Evidence to this effect is lacking in humans but has been observed in various mammalian species [reviewed in (H52)].

3.2. The δ Chain Variants

Table 7 lists six δ chain variants of which the structural abnormality has been determined. None of these residues are participating in contacts with heme or between chains and the variants are stable proteins. Most commonly observed is Hb-A_2'; a few homozygotes have been discovered (H21). The amount of the variant in the heterozygote is usually around 1% and the percentage of Hb-A_2 is decreased correspondingly. No normal Hb-A_2 is present in the homozygous Hb-A'_2 individual nor in the Hb-A'_2-Hb-Flatbush double heterozygote (L6). Hb-A_2-Babinga is found in the Babinga pygmies of the Central African Republic (J23) and in some American Negroes (H49). Hb-A_2-NYU has been observed in East Europeans of Jewish origin (H38, R12). A variant of Hb-A_2, present in Alberta Indians (V4), was recently identified as Hb-A_2-Spákia, which was discovered in a Cretan (J13); this observation might have some interesting anthropological consequences (H38).

TABLE 7
Variants of the δ Chain

Position[a]	Residue	Substitution	Name	References
2-NA2	His	→ Arg	A_2-Spákia	(J13)
12-A9	Asn	→ Lys	A_2-NYU	(R12)
16-A13	Gly	→ Arg	A'_2(= B_2)	(B10, J15)
22-B4	Ala	→ Glu	A_2-Flatbush	(J15, R9)
69-E13	Gly	→ Arg	A_2-Indonesia	(L27, L29)
136-H14	Gly	→ Asp	A_2-Babinga	(H49, J23)

[a] Refers to position in chain and to that in helix or interhelical section, respectively.

3.3. The γ Chain Variants

In 1968 Schroeder and co-workers (S22) described the presence of two types of γ chains in the normal human newborn which are the products of nonallelic structural genes (see Section 5). These two types of γ chains are termed $^{G}\gamma$ and $^{A}\gamma$ chains because position 136 is occupied by a glycyl residue in the $^{G}\gamma$ chain and by an alanyl residue in the $^{A}\gamma$ chain. Several γ chain variants have been described. The structural

TABLE 8
VARIANTS OF THE γ CHAIN

Name	Position[a]	Residue Substitution	Comment	References
F-Texas-1	5 (A2)	Glu → Lys	—	(J7, S6)
F-Texas-2	6 (A3)	Glu → Lys	—	(L5)
F-Alexandra	12 (A9)	Thr → Lys	—	(L33)
F-Jamaica	61 (E5)	Lys → Glu	Variant of $^{A}\gamma$ chain	(A3)
F-Malta-I	117 (G19)	His → Arg	Variant of $^{G}\gamma$ chain	(C7)
F-Hull	121 (GH4)	Glu → Lys	Variant of $^{A}\gamma$ chain	(S1)

[a] Refers to position in chain and to that in helix or interhelical section, respectively.

abnormality of six have been determined (Table 8) whereas the chemical variation in Hb-F-Roma (S45), Hb-F-Warren (H44), Hb-F-Houston (S5) and some other variants (S22) has not been elucidated. Some of these variants have abnormal $^{G}\gamma$ chains and others abnormal $^{A}\gamma$ chains.

3.4. DISTRIBUTION OF HEMOGLOBIN VARIANTS

Despite the discovery of over 150 human hemoglobin variants only a few, namely Hb-S ($\alpha_2\beta_2^{6\ Val}$), Hb-C ($\alpha_2\beta_2^{6\ Lys}$), Hb-$D_{Los\ Angeles}$ ($\alpha_2\beta_2^{121\ Gln}$), and Hb-E ($\alpha_2\beta_2^{26\ Lys}$), occur quite frequently in some parts of the world. Hemoglobin S is found in tropical Africa, the Mediterranean region, Southern Arabia, and India and in emigré populations. The incidence of the Hb-S trait in American Negroes is 8–9%; the highest incidence (some 40%) is found in parts of Africa and Greece. The distribution of the $Hb_{\beta S}$ gene corresponds well with that of falciparum malaria, and Hb-S provides indeed the classic example of how selective advantage leads to maintenance of a (disadvantageous) mutant gene at a high frequency. The presence of Hb-S likely inhibits multiplication of trophozoites, which keeps the parasite counts low and shortens the duration of the infection, thus markedly decreasing the mortality rate at a time when the individual does not yet have a resistance to malaria (A12).

High frequencies of the $Hb_{\beta C}$ gene are found in countries of West Africa, Ghana particularly, where in certain areas the incidence may approach the 20%. The variant is found in 1–2% of the American Negro population. No selective advantage for $Hb_{\beta C}$ has been demonstrated.

Hb-$D_{Los\ Angeles}$ (also often called Hb-D_{Punjab}) occurs rather commonly in northwestern India, where its frequency may be close to 3% in Sikhs of the Punjab.

Hb-E is found almost exclusively in southeastern Asia, notably in

Burma, Thailand, Cambodia, Vietnam, Laos, Malaya, and Indonesia. Its incidence among Chinese is low. The reason for the high frequency of Hb-E in these countries is not clear. The hemoglobinopathy causes a moderate anemia with microcytosis and targeting in the homozygote.

Reviews on hemoglobinopathies often contain a detailed description of studies pertaining to the distribution of hemoglobin variants; the most comprehensive review has been given by F. B. Livingstone (L30).

4. Thalassemia

4.1. Introduction and Classification

The term thalassemia refers to a group of disorders which result from a genetically determined reduction in the synthesis of hemoglobin polypeptide chains. The clinical picture of thalassemia was first described in 1925 (C29); hematological alterations concern changes in red cell morphology and lowering of red cell indices. Thalassemia is a collection of diverse entities which result from many distinct disorders in hemoglobin synthesis. Measurements of the rates of synthesis of globin polypeptide chains and clinical and hemoglobin characteristics, among others, have made it possible to classify the thalassemia syndromes more definitely into several types, such as the β-, δ-, $\beta\delta$-, and α-thalassemias. These forms will be discussed separately; some recent monographs and reviews should also be consulted (C24, F2, F8, F9, L3, L7, R35, S47, W9, W10, W11).

4.2. β-Thalassemia

Classical thalassemia, as originally described by Cooley and Lee (C29), is now known as β-thalassemia because its determinant is allelic or linked to the Hb_β structural locus and because the relative production of β chains is impaired.

4.2.1. *The Mediterranean β-Thalassemia Heterozygotes*

Patients with this abnormality suffer from mild hypochromic anemia with microcytosis and signs of hemolysis; a highly characteristic property is the 2- to 3-fold increase in the relative production of Hb-A_2. The Hb-level may vary between 7.5 and 11.5 g/100 ml. Anemia is the most common disturbance followed by hepatomegaly, gall stones, jaundice, splenomegaly, and others (G5). The red blood cell count varies between 5.5 and 7.5 million/mm^3 with mean cell volumes (MCV) of 55–80 μ^3 and mean cellular hemoglobin concentrations (MCHC) of 25–30%. The peripheral blood film shows hypochromia and microcytosis,

aniso- and poikilocytosis, target cells, basophilic stippling, and nucleated red cells. Osmotic fragility is decreased. The amount of Hb-A_2 may be as high as 9%, and the level of Hb-F is normal or elevated to about 10%.

4.2.2. *The Mediterranean β-Thalassemia Homozygote*

The diagnosis of classical thalassemia major is usually made during the first few months of life at the time in development when γ chain synthesis normally ceases. The hemoglobin level varies between 5 and 7 g/100 ml and may fall as low as 2 g/100 ml. Repeated blood transfusions are required, sometimes at weekly intervals. Microcytosis is extreme (MCV of 45–60 μ^3 with MCHC values of 20–30%), and a moderate reticulocytosis exists (5–20%). The peripheral blood film is most characteristic, with many small schizocytes and large macrocytes, marked aniso- and poikilocytosis, leptocytosis, ovalocytosis, basophilic stippling, nucleated red cells, Howell-Jolly bodies, Cabot rings, and mild leukocytosis. The synthesis of γ chains is continued but at a rate which is insufficient for a complete compensation of the decreased β chain synthesis. The amount of Hb-F is usually greater than 50% and values between 10 and 95% have been reported. The percentage of Hb-A_2 is normal or decreased.

4.2.3. *The African Variant of β-Thalassemia*

The incidence of heterozygous β-thalassemia among the American Negro is approximately 0.8% (G12). Subjects with this disorder have a Hb level of 10–13 g/100 ml, with MCV values of 70–75 μ^3 and MCHC values of 26–30%. Osmotic fragility of the cells is decreased. Diagnosis is usually an accidental finding or results from family studies.

Homozygosity for this type of β-thalassemia has seldom been observed (B69, B70, C33, H9, S32) although the probability of its occurrence is about 1 in 60,000. These patients show a mild clinical course with Hb levels between 8 and 11 g/100 ml, MCV values of 65–70 μ^3, and MCHC values of 25–30%. Osmotic fragility of the red cells is markedly decreased; reticulocytosis is mild (1–4%). Peripheral blood films show target cells, basophilic stippling, aniso- and poikilocytosis, hypochromia and microcytosis, and a few nucleated red cells. Over 50% of the hemoglobin is of the fetal type, and the Hb-A_2 level is at least twice the normal value (in some patients 8–10%). The mild clinical course together with the hemoglobin characteristics in these patients suggest that this variant is fundamentally different from that observed in other racial or ethnic groups.

4.2.4. *The Dutch Variant of β-Thalassemia*

In 1966 Schokker *et al.* (S14, W19) described two siblings having a homozygous β-thalassemia, their heterozygous parents (who are second cousins), and as many as twenty additional heterozygous relatives. The family is of Dutch origin. This Hb-F variant is characterized in the heterozygote by the production of 5–15% Hb-F and unusually high levels of Hb-A_2. Hb-A is absent in the two homozygotes, who produce 2–3% Hb-A_2 and 97–98% Hb-F. These homozygotes, about 40 years old at the time of study, are affected to an unusually mild extent by the disorder (Hb levels between 12 and 14 g/100 ml) but exhibit characteristic stigmata of β-thalassemia.

4.2.5. *The Silent β-Thalassemia Carrier*

In 1969 Schwartz (S29) described an Albanian family of two children with mild β-thalassemia major. Their mother had a classical β-thalassemia heterozygosity, but the father had a normal red cell morphology and normal levels of Hb-A_2 and Hb-F. The synthesis of β chains in this individual was impaired, although to a lesser extent than is usually observed in the classical β-thalassemia. Interaction of this type of β-thalassemia with the classical type results in a thalassemia major of reduced severity because the apparent homozygous children from this marriage are only mildly affected with levels of Hb-F of less than 12%.

4.2.6. *Combinations of β-Thalassemia with Hemoglobin Variants*

Reports describing patients with Hb-S-β-thalassemia or with Hb-C-β-thalassemia are numerous [(A5, A6, B28, C22, K21, K23, N12, R38, S50, S51, S63) and many others]. The patients suffer from a mild to rather severe anemia (Hb levels between 7.5 and 12 g/100 ml) which is usually microcytic and hypochromic. Hemoglobin analysis shows a high percentage of the abnormal variant (60–80%), elevated Hb-A_2 levels (4–7%), variable amounts of Hb-F (1–20%) and of Hb-A (0–20%). Patients who produce notable amounts of Hb-A are usually less severely affected than patients in which the normal Hb-A production is completely suppressed.

Hb-E-β-thalassemia is a rather common disorder among East Asians [(L19, N3, P25) and others]. Patients with Hb-E-β-thalassemia seem not to produce Hb-A; this suggests that only one type of β-thalassemia is present. The clinical picture is similar to that of classical β-thalassemia major.

Additional combinations include Hb-D-Los Angeles-β-thalassemia

(J6, S9), Hb-J-β-thalassemia (S68, W24), and Hb-G-β-thalassemia (S31).

4.2.7. *Synthesis of Polypeptide Chains in β-Thalassemia; Molecular Pathology*

A diminished synthesis of β chains by peripheral blood cells of β-thalassemia individuals has been established by different groups of investigators (B11, B12, B13, B14, B17, B18, B68, C20, C26, C27, H11, H13, K4, M23, M24, P20, S30, W13, W15, W18). The procedure consists of measuring the *in vitro* incorporation of radioactive amino acid (leucine-^{3}H) into the α and non-α chains of the hemoglobin by erythrocytes. Radioactive globin is separated into α and non-α chains by CM-cellulose chromatography and the specific activities of the separate chains compared (see Section 6). Figure 12, which is taken from reference (M7), presents some illustrative data. Figure 12A shows the elution pattern of the radioactive α and β chains of hemoglobin from cells of a nonthalassemic individual; Fig. 12B, that of a homozygous β-thalassemia patient; and Fig. 12C, that of a subject with Hb-S-β-thalassemia. The decreased synthesis of β chains is evidenced from the decreased radioactivity of the protein in the appropriate elution zones; the data on the Hb-S-β-thalassemia patient are particularly illustrative because of the high leucine-^{3}H uptake by the β^S chain in contrast to that of the β^A chain. The diagnosis of β-thalassemia can be confirmed by this type of measurement; the technique has even made it possible to diagnose homozygosity at time of birth (G1, K3).

The extent of the decrease in β chain synthesis is usually determined by the ratio between the specific radioactivities of the β chain and the α chain. In patients with a severe β-thalassemia this β:α ratio ranges from 0 to 0.30. Among these homozygous patients perhaps four subgroups can be recognized based on the amount of β chain produced relative to that of the α chain. Besides the group with no detectable β chain production, there is a group with low β chain production and a β:α ratio of about 0.10, a third group with a β:α ratio of about 0.20, and a fourth with a β:α ratio of about 0.30 (S29). Subjects with β-thalassemia trait usually show β:α ratios which are intermediate between those of the homozygous relatives and normal controls. This indicates that the depression of β chain synthesis is proportional to the gene dose.

Speculations as to the cause of the reduction in β chain synthesis have been many. It seems that this reduction is caused either by a defective mechanism of ribosomal assembly of (structurally normal) β chains due to an abnormal mRNA or by a decreased amount of normal mRNA.

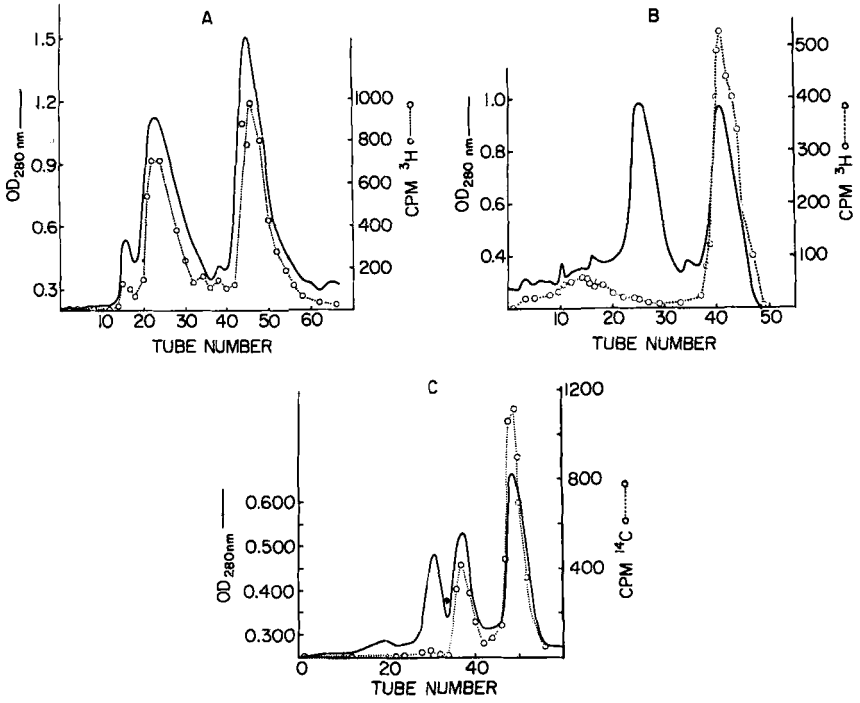

FIG. 12. The separation of globin chains prepared from cells incubated with leucine-^{3}H by chromatography on a column of CM-cellulose. Solid line represents the optical density at 280 nm, and the dotted line, the radioactivity. (A) A normal subject; the β chain is eluted in tubes 20–30 and the α chain in tubes 42–52. The α:β ratio of radioactivity is 1.0. (B) A patient with β-thalassemia major; the γ chain is found in tubes 10–20, the β chain in tubes 22–32, and the α chain in tubes 38–50. The α:β ratio of radioactivity is greater than 50. (C) A patient with Hb-S-β-thalassemia; the normal β chain is found in tubes 25–33, the β^S chain in tubes 35–42, and the α chain in tubes 46–55. The α:β ratio of radioactivity is 2.9. From Marks and Bank (M7) with permission of the authors and publisher.

Most recent data (N13, S30) suggest that the defect in β-thalassemia is not in the ability of the thalassemic ribosomes to translate normal mRNA but in the mRNA itself. It is possible that either normal mRNA is present in reduced amount or that an alteration in its nucleotide sequence affects the rate of translation.

The defect also results in an excess synthesis of α chains. These chains precipitate and are observed as inclusion bodies. Cells with these precipitates are predisposed to a preferential destruction in the bone marrow or the peripheral circulation and a characteristic hemolytic anemia will result.

4.2.8. *Distribution of β-Thalassemia*

Studies describing the incidence of β-thalassemia in various parts of the world are too many to be discussed in this review. This short summary is primarily taken from the review published in 1966 by Rucknagel (R35). Frequency data refer to those given for heterozygotes.

a. Mediterranean basin. Frequency in Italy varies with about 20% in the Po Valley, 3 to 13% in Sicily, and as much as 38% in some villages in Sardinia. In Greece it varies from 6 to 14%. Data are available also from Turkey, Spain, Portugal, and France. The incidence in Egypt is low (less than 1%), but varies between 1 and 15% in the Berbers of Algeria.

b. Africa. The incidence in the African Negro is low and does probably not exceed the 2%; the American Negro shows a frequency of 0.5–1.0%.

c. Middle East. The disorder is found in Moroccans, German Jews, Kurdish Jews (incidence may be as high as 20%), in Lebanon, Syria, and Arabia.

d. Far East. In Thailand the overall frequency is about 5%. Similar or lesser frequencies have been reported from Southern China, New Guinea, South Vietnam (about 2%), Philippines (about 1%). Frequency data for India are not available, but the occurrence of the disorder is well documented. The type of thalassemia found in these populations is the one in which the β chain production is completely suppressed.

e. Others. β-thalassemia is probably absent in North and South American Indians. The thalassemia observed in these continents seems to be due to immigration from Europe and Asia. The incidence among North Europeans is extremely low.

4.3. βδ-Thalassemia

This type of thalassemia, also called type II β-thalassemia or F-thalassemia, is a relatively rare disorder. It is characterized by similar hematological alterations as found in β-thalassemia. However, the heterozygote does not have the increase in the level of Hb-A_2 but instead produces significant quantities of Hb-F (5–15%). The disorder was first described in 1961 (Z4) and has primarily been found in Greeks and Italians (C25, G2, M3, S59). Homozygotes for F-thalassemia produce Hb-F only; thus, β and δ chains are not produced (B66, R8, S48). Hemoglobin A is also not observed in the patient with a double heterozygosity for Hb-S and F-thalassemia (S58). Homozygotes are on the average somewhat milder affected than β-thalassemia homozygotes; they are less anemic and have usually a lesser transfusion requirement. The

same is true for subjects with a double heterozygosity for F-thalassemia and the classical β-thalassemia (S59). The present concepts on the pathogenesis of thalassemia probably cannot account for the findings in $\beta\delta$-thalassemia.

4.4. δ-Thalassemia

Homozygosity for this type of thalassemia results in a complete suppression of δ chain production, and thus in a complete absence of Hb-A_2 (F5, O2). Heterozygotes have decreased levels of Hb-A_2 (1.2–1.6%). Subjects with this anomaly are free of clinical symptoms, have a normal red cell morphology, and a normal osmotic fragility of the erythrocytes.

4.5. γ-Thalassemia

The existence of a γ-thalassemia has not definitely been established although a preliminary report has appeared describing a striking defect in γ chain synthesis in a newborn (K5). The genetic heterogeneity of γ chain production (see Section 5) suggests that such a thalassemia should be relatively mild because reduction of γ chain synthesis will affect only one of the nonallelic Hb_γ structural loci. As stated by Stamatoyannopoulos (S57), detection of such a condition should be possible with hematological techniques, but characterization of the type of γ-thalassemia, i.e., suppression of either the Hb_{G_γ} or the Hb_{A_γ} loci, will require chemical analysis of the Hb-F that is produced. The condition probably will cause a mild neonatal anemia, which will correct itself when the production of γ chain ceases and is replaced by that of the β and δ chains.

4.6. α-Thalassemia

Basic information on α-thalassemias, i.e., defects with an impaired production of α polypeptide chains, is still limited because of difficulties in identifying the simple adult heterozygote. Detection of α-thalassemia can best be made by hemoglobin analysis of cord bloods and of subjects with Hb-H disease.

4.6.1. *α-Thalassemia Heterozygosity*

Detailed hematological studies of several hundred obligatory adult heterozygotes for α-thalassemia suggest that this condition is often associated with a normal blood picture (P23). The hemoglobin concentration varies between 10 and 13 g/100 ml with MCV values of 60–80 μ^3 and MCH of 22–30 pg per cell; hypochromia is not prominent and reticulocytosis is hardly observed. Osmotic fragility of red cells is usually reduced.

Far more reliable information comes from analyses of cord blood samples because of the presence of significant amounts of Hb-Bart's. This variant has been identified as a fetal hemoglobin without α chains and consisting of (four) γ chains only (H62). Hb-Bart's or γ_4 has a 10- to 12-fold increase in the affinity for molecular oxygen, which makes it difficult to release the oxygen to tissues (H22). The incidental occurrence of γ_4 hemoglobin in cord bloods of newborns was first observed in 1957 (F3), and from the beginning it has been considered likely that the occurrence of Hb-Bart's was associated with a decreased synthesis of α chains because of the red cell morphology of the individual carrying it (I4).

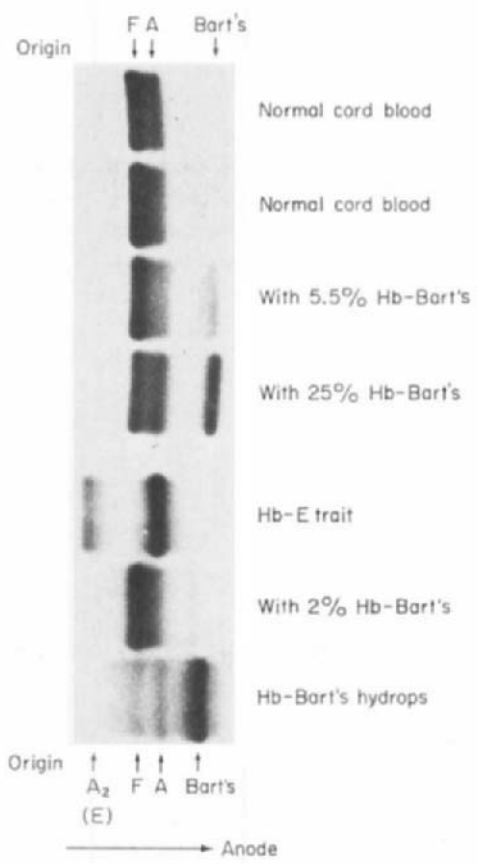

FIG. 13. Starch gel electrophoresis of hemoglobin of cord blood samples from newborns with various types of α-thalassemia. Tris–EDTA–boric acid buffer, pH 8.6. Stained with *o*-dianisidine. From Pootrakul *et al.* (P22) with permission of the authors and publisher.

Small amounts of γ_4 have been found in apparently healthy newborns of different ethnic groups—for instance, in Thailand, China, Indonesia, Malaya. Various papers present such data as frequency data for α-thalassemia; however, the use of less sensitive methods makes this information often unreliable (N5). It seems that best results are obtained by starch gel electrophoresis using a Tris–EDTA–boric acid buffer, pH 8.9 (E3). Figure 13 presents examples of separations observed in a survey of cord blood samples from 1408 newborn babies in Bangkok, Thailand (N5). The results fall in five distinct groups, namely: no Hb-Bart's; about 2% Hb-Bart's; about 5% Hb-Bart's; about 25% Hb-Bart's; and 80–90% Hb-Bart's [(N5, N6, P21, P22) and others]. The amount of Hb-Bart's seems directly related to the severity of the α-thalassemia. The incidence of Hb-Bart's in these 1408 samples was over 20% (N5). Observations such as these are taken to indicate that more than one type of α-thalassemia exist. The first, α-Th_1 or the classical form, is considered to be the severe α-thalassemia allele which in the heterozygous newborn causes the presence of some 5% Hb-Bart's. The second, α-Th_2, is a milder form which will lead to the presence of about 2% Hb-Bart's in the heterozygous newborn. This basic assumption, which was already suggested over 10 years ago from studies of subjects with Hb-H disease [(H28, K19, M28, W4, W8) and others], will also explain the additional observations; cord blood samples from newborns with a homozygosity for type α-Th_1 will have 80–90% Hb-Bart's, those with a homozygosity for type α-Th_2 will have some 5% Hb-Bart's, whereas the double heterozygous baby, α-Th_1/α-Th_2, will produce some 25% Hb-Bart's and will develop Hb-H disease.

4.6.2. *α-Thalassemia Homozygosity*

The homozygous state of α-thalassemia type-1 (α-Th_1) leads to a form of erythroblastosis fetalis and intrauterine death [(K2, L18, L20, L21, L22, L23, L24, L26, P4, T6, T7, T8) and others]. This form of hydrops fetalis is found in Chinese from various countries, in Filippinos, and in Thais. All children are either stillborn or die within hours after birth. The hematological observations usually include anemia with reticulocytosis, hypochromia with macrocytosis, aniso- and poikilocytosis, and many erythroblasts. The red cells sickle rather easily. Most fetuses are hydropic (but not all).

The hemoglobin consists mainly of Hb-Bart's (γ_4) and some Hb-H (β_4). Some papers (L18, L22) report the presence of a small amount of normal Hb-A, but this conclusion is based on electrophoretic mobilities only. More recent studies (T8) have offered evidence for the pres-

ence of some 80% γ_4, of very small amounts of β_4, and of a third component (some 15–20%) which is identical to Hb-Portland-1 or $\gamma_2\zeta_2$ (see Section 2). The absence of α chain containing hemoglobins, such as Hb-A, Hb-F, and Hb-A_2, shows that the production of α-polypeptide chains is completely suppressed and confirms the hypothesis that these babies are suffering from homozygous α-thalassemia of the complete type (α-Th_1).

Homozygosity for the α-thalassemia of the incomplete type (α-Th_2) does not seem to cause ill effects. It results in a phenotype which is more or less identical to the α-Th_1 heterozygosity (P22).

4.6.3. *Hemoglobin H-Disease*

This disorder was first described in 1955 in a Chinese family by Rigas *et al.* (R21, R22, R23) and independently by Gouttas *et al.* (G14). The condition has been observed in Chinese [(D13, R21) and others], Italians [(A7, B76, D6, N8) and others], Turks (G11, I2), Iranians (R3), Arabs from Kuwait (A8), Jews (C23, R7), Gurka's (B65), Melanesians and Hawaiians (J9), Swedes (H7), Germans (R25), Dutch (B55), and of course the Thais (N4, W3). Clinically, hemoglobin-H disease is a relatively mild thalassemia ("thalassemia intermedia"), but the manifestations may vary greatly. The presenting features include splenomegaly and intermittent bouts of hemolysis. A moderate anemia persists with reticulocytosis, aniso- and poikilocytosis, hypochromia and decreased osmotic fragility (N4, W3). The formation of erythrocyte inclusion bodies after supravital staining with brilliant cresyl blue is a diagnostic feature of the disease.

The major hemoglobin variant in subjects with this condition is Hb-H. Hb-H consists solely of four β chains and its gross structure is β_4 (J11). The variant is heat labile, rather acid resistant (B34), unusually susceptible to oxidative denaturation, and has a 10- to 12-fold increase in affinity for molecular oxygen (B27). The proportion of Hb-H in cases with Hb-H disease ranges from 2 to 40%; the accuracy of these data is questionable because of difficulties in quantitating this unstable variant.

The newborn with Hb-H disease produces about 25% Hb-Bart's and only small quantities of Hb-H. This γ_4 is slowly disappearing during the first 6 months of life. Small amounts of a δ_4 component have also been isolated from blood of adult patients with Hb-H disease (D3). The clinical, biochemical, and hematological manifestations are indeed consistent with the hypothesis that Hb-H disease results from a double heterozygosity for the α-Th_1 and the α-Th_2 genes.

4.6.4. *α-Thalassemia in the Negro*

Small quantities (less than 1%) of Hb-Bart's are often observed in blood from Negro newborns (H10, H22, S3); the incidence approaches the 20%. In some babies a larger amount (up to 5%) is present. The α-chain deficiency does not express itself beyond infancy. Because Hb-H disease and hydrops fetalis have not been found in the Negro, it seems that either the α-Th_2 type or an additional type of α-thalassemia is present. The babies with the higher amounts of Hb-Bart's are considered to be homozygous for the deficiency and those with the minute amounts heterozygous.

4.6.5. *α-Thalassemia in Combination with β and α Chain Variants, and with β-Thalassemia*

α-Thalassemia in association with α chain variants has been found in Orientals [Hb-Q-α-thalassemia (L25, V6)] and in a Negro family [Hb-I-α-thalassemia (A13)]. In subjects with the first combination no Hb-A is present but only Hb-Q, Hb-H, and Hb-Bart's; thus, complete suppression of the normal α chain synthesis occurred. In the subjects with Hb-I-α-thalassemia some Hb-A was found in addition to large quantities of Hb-I.

The combination of α-thalassemia with β chain variants has frequently been observed; when associated with Hb-E a well-defined clinical syndrome is present which is primarily found in Thailand (T11, T13, W5). The combination of two α-thalassemia genes (α-Th_1 and α-Th_2) and a single Hb_{β^E} gene results in a thalassemia intermedia with moderate anemia and hepatosplenomegaly. At time of birth these patients produce some 25% Hb-Bart's, Hb-A, Hb-F, and small amounts of Hb-E. At a later age the disease is characterized by the presence of an increased γ chain production and a decreased β^E chain production; the hemoglobin phenotype shows some 15% Hb-E, 5–15% Hb-Bart's, and the remainder Hb-A. Hb-H (β_4) is hardly present as is the tetramer of the β^E chain.

Persons homozygous for the Hb_{β^E} gene and heterozygous for the α-Th_1 gene resemble patients with homozygous Hb-E disease except for a possible increase in Hb-F. Newborns with this combination have a considerable amount of Hb-Bart's. In subjects who are heterozygous for Hb-E as well as for either the α-Th_1 or the α-Th_2 gene the amount of β chain variant is lower than that found in simple Hb-E heterozygotes (T11). It has been suggested that in an α chain deficiency normal β chains might be bound in preference to abnormal β chains (T13).

Combinations of homozygous Hb-S disease and α-thalassemia have

also been described (A4, W16). This sickling disorder occurs mainly among Arabs and is associated with unusually high levels of fetal hemoglobin and a milder clinical condition.

α-Thalassemia heterozygosity in combination with heterozygous β-thalassemia gives the hematologic picture of thalassemia minor with intermediate values of Hb-A_2 (P2). Subjects with one α-Th_1 gene and two β-thalassemia genes have a mild form of Cooley's anemia (K6). The decreased severity of the condition has been explained by assuming that the imbalance of the α and β chain production is favorably influenced in this combination.

4.6.6. *Synthesis of Polypeptide Chains in α-Thalassemia*

Data from measurements of the rate of incorporation of leucine-^{14}C into α and β chains of hemoglobin in peripheral blood samples have shown that the rate of α chain synthesis is indeed depressed in various α-thalassemia disorders while that of the β chain remains normal (C19, K4). Kan *et al.* (K4) reported these α:β specific activity ratios: normals 1.02 ± 0.07; α-Th_2 heterozygosity 0.82–0.95; α-Th_1 heterozygosity 0.77 ± 0.05; hemoglobin H disease 0.41 ± 0.11. These data illustrate conclusively the significant difference in expression between the two α-thalassemia genes.

4.6.7. *Some Genetic Considerations*

Despite considerable progress, the genetics of the α-thalassemia is still uncertain. The main uncertainty centers around a possible multiplicity of the Hb_α structural locus (see Section 2.3.2). Assuming the existence of two nonallelic Hb_α structural genes such a concept permits a grouping of the α-thalassemias into several types (K7, K8, L8, L11). It is assumed that four genes (two homologous loci) are responsible for the synthesis of α chain. Because each of these genes can be "affected" in a similar fashion the augmented expression of α-thalassemia from "silent" to "classical" to "Hb-H disease" to "hydrops fetalis" is due to an increasing number of affected genes. However, according to calculations by Koler *et al.* (K20) and Wasi (W2), all data presented in the literature fit a one-locus model with two alleles as well as a two loci model without linkage. Required are more refined tests that allow differentiation between the various genotypes, for instance, by a careful determination of α:β specific ratios of the hemoglobin that is synthesized or by an improved quantitation of Hb-Bart's at time of birth. Moreover, a careful reexamination of certain families, such as that with members with α-thalassemia-Hb-Q_α disease, is required. The absence of Hb-A in subjects with this combination, for instance, can readily be explained by the

one-locus model, but the two-loci model requires an α-thalassemia gene closely linked to that of Hb-Q_α and the presence of such a combination might be difficult to prove.

An additional complication is the recent discovery of one, or more than one, silent Hb_α structural locus in chimpanzees and gorillas. The products of these genes, which are observed as hemoglobin in minute quantities, differ from the major α chain by several amino acid changes (B63). The possible existence of similar silent structural loci in man would open new genetic avenues for an understanding of α-thalassemia. It is of great interest that the production of a minor hemoglobin abnormality, Hb-Constant Springs, which has elongated α chains (see Section 3.1.9) is greatly increased in patients who have the characteristics of Hb-H disease (E4). These observations likely place this anomaly outside the current concepts of the genetic control of α-thalassemia; the determinant for this specific α chain might well be a silent nonallelic Hb_α locus which is activated in certain α chain deficiencies.

4.6.8. *Distribution of α-Thalassemia*

The global distribution of α-thalassemia is not easy to assess because of the difficulties in defining α-thalassemia heterozygosity in the adult. Most of the available data originate from analyses of subjects with Hb-H disease, hydrops fetalis, and related conditions. As indicated previously, the highest incidence is in East Asia (Thailand, Malaysia, South Vietnam, Indonesia), Southern Europe (Sardinia, other parts of Italy, Greece, Turkey), West Africa, and, to a much lesser extent, the Middle East and European countries.

4.6.9. *α Chain Deficiencies in Acquired Disorders*

Hemoglobin H has been observed in association with some forms of leukemia, mostly erythroleukemia or Di Guglielmo's syndrome (B22, B30, R32, W22). In these cases, as in Hb-H disease, the excess β chains form the tetrameric Hb-H molecule. The suppression of α chain synthesis might well be due to a yet undetectable chromosomal abnormality affecting the Hb_α locus or loci (R32).

5. The Genetic Heterogeneity of Fetal Hemoglobin

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Chemical analyses of Hb-F from newborn infants have shown that this protein is a mixture of two components with γ chains that differ in residue 136 of the γ chain (S22). The chemical examination of Hb-F re-

quires treatment with cyanogen bromide to cleave the α and γ chains at methionyl residues and to form six cyanogen bromide (CB-) peptides. The carboxy terminal fragment of the γ chain, termed γCB-3, is a tridecapeptide which can easily be separated from the five larger peptides by chromatography on columns of Bio-Gel P-100 and further purified by Dowex 1 chromatography (S22). This peptide has the sequence:

135 140 145
Val-Thr-Gly(or Ala)-Val-Ala-Ser-Ala-Leu-Ser-Ser-Arg-Tyr-His

The presence of a glycyl and an alanyl residue in position 136 indicates two peptides, and thus two γ chains; the chain with a glycyl residue in position 136 is termed the $^{G}\gamma$ chain and the one with an alanyl in this position the $^{A}\gamma$ chain.

Amino acid analysis reveals whether the γCB-3 peptide derives from the $^{G}\gamma$ or the $^{A}\gamma$ chain or from both. Calculation of the number of glycyl and alanyl residues will indicate the presence of $^{G}\gamma$ chains only when the values are 1 and 2, respectively (the two additional alanyl residues originate from positions 138 and 140), the presence of $^{A}\gamma$ chains only when the values are 0 and 3, respectively, and the presence of both chains when the values are nonintegral. The ratio of the two chains is most easily determined by using the glycine value as indicator; thus if Gly = 0.75 the ratio of the $^{G}\gamma$ and $^{A}\gamma$ chains is 3:1. Presentation of all data in this section will follow this procedure.

Similar data can also be obtained through amino acid analysis of the γT-15 peptide which can be isolated from tryptic digest of γ chains by a combination of paper electrophoresis and chromatography (C7).

5.1. Heterogeneity of Hb-F in the Newborn

5.1.1. *Normal Newborns*

The $^{G}\gamma$ to $^{A}\gamma$ ratio has been determined in the Hb-F of 108 newborn children from different racial and/or ethnic origin and all newborns had the two types of γ chain (S28). The average glycine value was 0.71 with a range of 0.52–0.88; the data presented in Fig. 14 show that the chemical heterogeneity of Hb-F is indeed a worldwide phenomenon. Because no newborn has either $^{G}\gamma$ or $^{A}\gamma$ chains alone, heterozygosity at a single Hb_{γ} structural locus is excluded as an explanation of the phenomenon.

5.1.2. *Newborns Heterozygous for γ Chain Variants*

Table 8 (Section 3) lists the several variants of which the structural abnormality has been determined. In addition, there are some abnormal fetal hemoglobins which are incompletely characterized. Figure 15 illus-

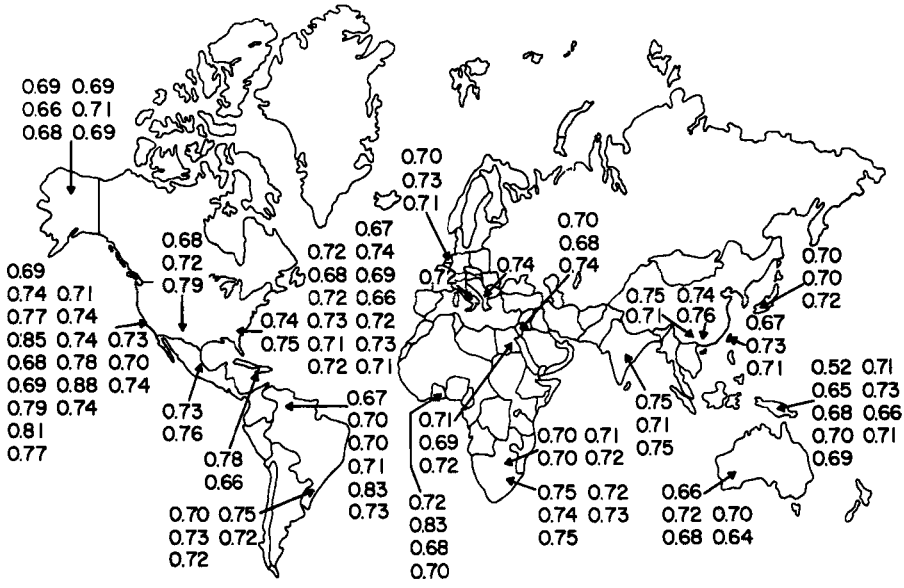

FIG. 14. Worldwide distribution of the genetic heterogeneity of the γ chain of Hb-F in the newborn. Infants of the following racial and/or ethnic groups have been studied: Caucasian, Negro, Eskimo, South and North American Indian, Egyptian, Jewish, Indian, Chinese, Japanese, Australian, and New Guinean aborigines. Figures indicate glycine value.

trates the electrophoretic mobilities of some variants. Data from chemical analyses of some variants indicate that position 136 of the abnormal γ chain is occupied by either a glycyl or an alanyl residue (A3, C7, S22). Thus, the variants are the product of an allele of either the Hb_{G_γ} locus or of the Hb_{A_γ} locus. These observations are convincing proof of the presence of multiple nonallelic Hb_γ structural genes and exclude ambiguous biosynthesis as an underlying cause of the chemical heterogeneity of Hb-F.

The amount of γ chain variants places them in four distinct groups (Fig. 16): (1) Some $^{G}\gamma$ chain variants, such as Hb-F-Malta-I, amount to about 22.5% (expressed as percent abnormal Hb-F of the total amount of Hb-F) or an $F_X:F_O$ ratio of about 1:3; (2) other $^{G}\gamma$ chain variants, such as an unknown variant found in a few Negro newborns, are present to about 13.5%, or an $F_X:F_O$ ratio of approximately 1:7; (3) some $^{A}\gamma$ chain variants, such as Hb-F-Hull and Hb-F-Jamaica, present to about 12.5% and likewise an approximate $F_X:F_O$ ratio of 1:7; (4) other $^{A}\gamma$ chain variants, such as Hb-F-Malta-II, amount to about 5%, or an $F_X:F_O$ ratio of about 1:19. The Hb-F-Malta-II variant has

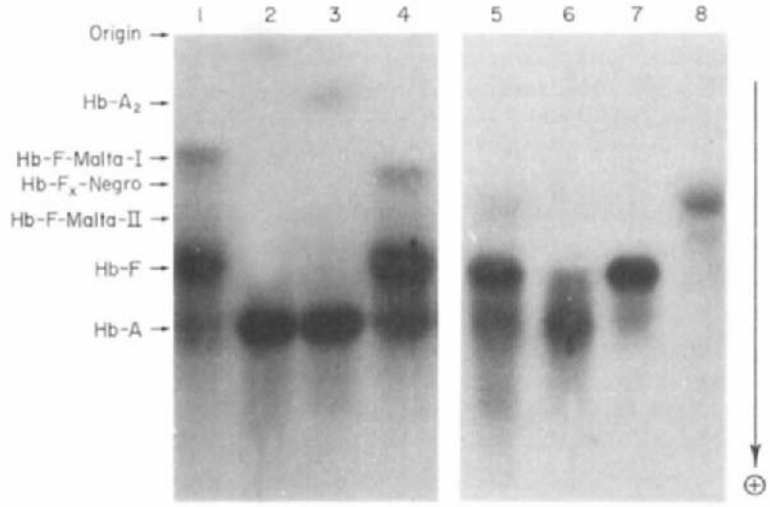

FIG. 15. Horizontal starch gel electrophoresis to show the small differences in mobility between three fetal hemoglobin variants. Left to right: (1) Hb-F-Malta-I heterozygote, (2) and (3) normal adults; (4) Hb-Fx in a Negro newborn; (5) Hb-F-Malta-II heterozygote; (6), (7), and (8), Hb-A, Hb-F, and Hb-F-Malta-II fractions, respectively, isolated by column chromatography on CM-cellulose. Tris–EDTA–boric acid buffer, pH 9.0, *o*-dianisidine stain.

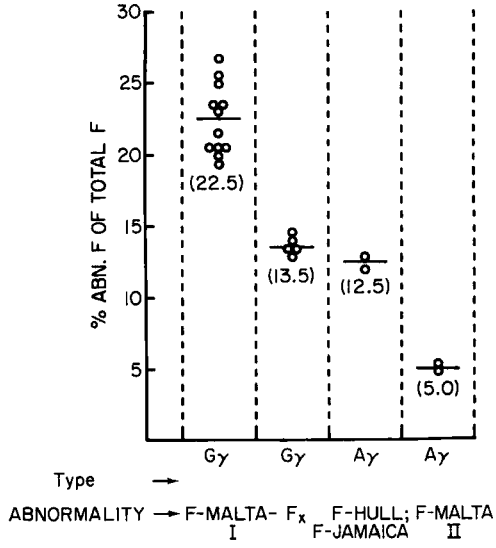

FIG. 16. The relative amounts of fetal hemoglobin variants which are abnormal in γ chains. Values are from individual heterozygotes aged 1–120 days. Numbers in parentheses are the mean values for the four groups.

been found thus far in three newborns and has not yet been characterized (H58). Other abnormal fetal hemoglobins which are present to the extent of 12–17% (F4, H44, J7, L5, L33, S8, S45) cannot be placed in the above groups because the nature of the residue at position 136 has not been defined.

5.1.3. *The Number of γ Chain Structural Genes*

Regardless of whether the γ chain variants that have been described above are mutants of the Hb_{G_γ} or H_{A_γ} genes, the amount that is produced varies severalfold. This result may be due to some unknown factor that controls the rate of synthesis, but it may also be due to multiple loci that produce at different rates. The subject of the number of γ chain structural genes has been discussed previously (S22); on the basis of the ratio of $^{G}\gamma$ and $^{A}\gamma$ chains in the newborn (about 3:1), there may be three $^{G}\gamma$ and one $^{A}\gamma$ genes ($^{G}\gamma^{G}\gamma^{G}\gamma^{A}\gamma$) all of which produce equal gene products. Substantiating evidence for this idea has not been forthcoming, and, indeed, contradictory evidence comes from the 2:3 ratio of $^{G}\gamma$ to $^{A}\gamma$ chains in the adult (see Section 5.2). However, a rather different hypothesis can be proposed if it is assumed that the varied amounts of abnormal fetal hemoglobins reflect the actual quantity of gene product not only of the mutated gene but also of the normal locus. Thus, on the basis of the quantities of the γ chain variants as presented in the preceding section, the presence of two $^{G}\gamma$ and two $^{A}\gamma$ genes is assumed. Each $^{G}\gamma$ pair produces identical $^{G}\gamma$ chains but in a ratio of about 2:1. These are designated the $Hb(^{G}_{m}\gamma)$ and $Hb(^{G}_{l}\gamma)$ loci where the prefixed subscripts *m* and *l* designate the more active gene and the less active gene, respectively. Likewise, the $Hb(^{A}_{m}\gamma)$ and $Hb(^{A}_{l}\gamma)$ genes produce in a ratio of about 2:1. Because the production of the $Hb(^{G}_{l}\gamma)$ and $Hb(^{A}_{m}\gamma)$ genes is about equal, products of the $Hb(^{G}_{m}\gamma)$, $Hb(^{G}_{l}\gamma)$, $Hb(^{A}_{m}\gamma)$, and $Hb(^{A}_{l}\gamma)$ genes are in the approximate ratio 4:2:2:1. Therefore, at birth the expected ratio of $^{G}\gamma$ to $^{A}\gamma$ chains should be 0.67 and is in substantial agreement with an average value of 0.71 in 108 newborn children. Substantiating evidence for this hypothesis may come from careful study of many more abnormal fetal hemoglobins. However, most direct evidence probably would be obtained from a homozygote for a γ chain variant; the likelihood of detecting a homozygote is slight except in the case of F-Malta-I which has an incidence of about 2% in newborns on this island (C7).

5.1.4. *Infants during the First Year of Life*

The Hb-F which constitutes about 75% of the total hemoglobin in cord blood samples of full-term newborns (see references in O3) is

gradually replaced by Hb-A and Hb-A_2 in a process that is essentially complete in 150 days after birth (K13). Serial examinations of Hb-F from newborns in the first 7 months of life have shown that the ratio of $^{G}\gamma$ to $^{A}\gamma$ decreases from the average glycine value of 0.71 at time of birth to about 0.4 by 5 months of age (S23, S27). This last value approximates that found for the small amounts of Hb-F found in the normal adult (S27). The change becomes apparent between the second and third months of life when postnatally synthesized Hb-F constitutes an appreciable percentage of the total Hb-F. These data indicate a complex regulatory mechanism that is operative in the switch from γ chain to β and δ chains. Two features, namely, one factor which permits production of Hb-A and Hb-A_2 and suppresses that of Hb-F, and a second which controls the ratio of the $^{G}\gamma$ and $^{A}\gamma$ chain production, may be active and may interact with each other.

5.2. Heterogeneity of Hb-F in the Normal Adult

Because Hb-F approximates only about 0.5% of the total hemoglobin in adults (S26), chemical analyses of Hb-F in normal adults are hampered by difficulties of isolation. Data from analyses of Hb-F of eight adults show an average value for Gly = 0.38 with a range of 0.23–0.55 (S27).

5.3. Heterogeneity of Hb-F in the Hereditary Persistence of Fetal Hemoglobin (HPFH)

This anomaly was described first in 1955 in the Negro (E1), and has later been observed in Greece (F6), in the Far East (W6) and in other ethnic groups [(B43) lists an extensive bibliography]. The condition in its heterozygous form is characterized by a familial persistence of about 5 to 40% Hb-F in adult life, absence of hematological abnormalities, normal or decreased levels of Hb-A_2, and a more or less uniform distribution of Hb-F throughout the red cells. Individuals who are heterozygous for the Negro type of HPFH as well as for a β chain variant fail to make Hb-A [(C28, T5) and references quoted in W6]. The four known homozygotes for the HPFH condition make Hb-F only (R24, S43, W21). Although the HPFH condition is relatively homogeneous in its clinical and hematological symptoms, heterozygeneity exists at the molecular level. Structural analyses of Hb-F of over 100 cases with this condition distinguish three major catagories (H50, H52, H53, H54, H57, S24) as follows: (A) the Hb_{G_γ} class: Hb-F with $^{G}\gamma$ chains only; (B) the Hb_{A_γ} class: Hb-F with $^{A}\gamma$ chains only; (C) the $Hb_{G_\gamma}Hb_{A_\gamma}$ class: Hb-F with $^{G}\gamma$ and $^{A}\gamma$ chains.

5.3.1. *The Hb_{G_γ} Class of HPFH*

This condition has been observed in a few Negro families and in two Indian families from the area of Bombay. One Indian is a homozygote and produces only Hb-F of the $^{G}\gamma$ type (S65). Homozygosity for this condition results in a moderately severe hematological disorder which resembles a mild form of thalassemia, because the 9-year-old homozygote suffers from a hemolytic anemia with splenomegaly, and requires an occasional blood transfusion. Heterozygotes in the Hb_{G_γ} class of HPFH fall into two groups on the basis of the percentage of Hb-F. Table 9 presents distinct features of these subgroups.

5.3.2. *The Hb_{A_γ} Class of HPFH*

This category has been found in members of three Greek families (H54) and in one additional Caucasian family (H53). Only heterozygotes have been observed, but the Greek form of HPFH was also found in three individuals in conjunction with β-thalassemia. The condition does not cause distinct clinical and hematological symptoms. The two forms of the Hb_{G_γ} class are also differentiated by the percentages of Hb-F (Table 10). Of fundamental importance is indirect evidence for the

TABLE 9
THE $Hb_{G\gamma}$ CLASS OF HPFH HETEROZYGOTES

Criterion	In two families from India	In three Negro families	In one Negro family
Red cell morphology	Normal	Normal	Normal
MCV	Decreased	Slightly decreased	Normal
MCH	Decreased	Normal	Normal
Distribution of Hb-F[a]	Approx. uniform	Approx. uniform	Approx. uniform
Percent Hb-A_2[a]	Decreased	Decreased	Normal
Percent Hb-F[a]	8–20	9–22	2–7
Production of β chains[b]	Absent *in cis*	Absent *in cis*	Unknown
Gly in γCB-3[c]	0.99–1.13	0.92–1.15	0.88–1.01[d]
Group designation (H56, S65)	Group 1b	Group 1b	Group 1a

[a] Consult Section 6 for methodology.

[b] From observations made in subjects with a heterozygosity for the $Hb_{G\gamma}$ type of HPFH and for the β chain variants Hb-E or Hb-S.

[c] Some values are slightly higher than the theoretical value of 1.0 because of the presence of extraneous glycine.

[d] Small amount of normal Hb-F (with $^{G}\gamma$ and $^{A}\gamma$ chains) is likely a significant portion of Hb-F; the slightly lower values are due to the presence of $^{A}\gamma$ chains which are produced by the normal chromosome.

TABLE 10
THE $Hb_{A\gamma}$ CLASS OF HPFH HETEROZYGOTES

Criterion	In families from Greece	Family described in reference (H53)
Red cell morphology	Normal	Normal
MCV	Normal	Normal
MCH	Normal	Normal
Distribution of Hb-F[a]	Approx. uniform	Approx. uniform
Percent Hb-A_2[a]	Decreased	Normal
Percent Hb-F[a]	16.5–19	4–7
Production of β chains	Probably present *in cis*[b]	Unknown
Glycine in γCB-3[c]	0.06–0.13	0.07–0.15
Group designation (H56, S65)	Group 2b	Group 2a

[a] Consult Section 6 for methodology.
[b] Indirect evidence from reference (F7).
[c] Values are slightly higher than the theoretical value of 0.0 because of the presence of extraneous glycine.

possible presence of active Hb_β and Hb_δ loci *in cis* to the HPFH determinant (F6).

5.3.3. *The $Hb_{A_\gamma} Hb_{G_\gamma}$ Class of HPFH*

The majority of patients with HPFH fall in this category. Although heterogeneity is observed, three groups may be distinguished (Table 11). The majority of heterozygotes fall in group 3b; the average glycine value is 0.39 and Hb-F is about 25%. Group 3c (2 families) has about the same amount of Hb-F but the average glycine value is increased to 0.61. Sub-

TABLE 11
THE $Hb_{A\gamma}$ $Hb_{G\gamma}$ CLASS OF HPFH HETEROZYGOTES

Criterion	(a)	(b)	(c)
Red cell morphology	Normal	Normal	Normal
MCV	Normal	Normal	Normal
MCH	Normal	Normal	Normal
Distribution of Hb-F[a]	Approx. equal	Approx. equal	Approx. equal
Percent Hb-A_2[a]	Decreased	Decreased	Decreased
Percent Hb-F	11–25	16–32	22–32
Production of β chains[b]	Unknown	Absent *in cis*	Absent *in cis*
Glycine in γCB-3	0.09–0.29	0.26–0.51	0.57–0.69
Group designation (H56, S65)	Group 3a	Group 3b	Group 3c

[a] Consult Section 6 for methodology.
[b] Evidence from subjects with HPFH-heterozygosity together with a heterozygosity for Hb-S or Hb-C.

jects of two families which form group 3a produce less Hb-F (mean value 17%) and the average glycine value is also decreased.

No patient exhibits any significant hematological abnormality, the Hb-A_2 levels are usually decreased, and no Hb-A is present if a β chain abnormality hemoglobin is produced *in trans* (detected only in subjects of groups 3b and 3c). The cellular distribution of Hb-F is approximately uniform.

Data on two homozygotes are available (H57, S43); the glycine values (0.52 and 0.59) are slightly higher than those of heterozygous relatives which belong to group 3b.

5.3.4. *HPFH–β Chain Variant Combinations*

When an allele of the Hb_β locus is present *in trans* to the HPFH determinant, the production of γ chains is increased. In subjects of group 1b, the Hb-F is doubled or tripled when either a β^S or β^E heterozygosity is also present; the increase in γ chain production concerns that of $^G\gamma$ chain only because the glycine values in the double heterozygotes is the same as in the simple heterozygotes (S65). When, in subjects of groups 3b and 3c, an Hb_{β^S} locus is *in trans* to the HPFH determinant, Hb-F is increased from about 25% to 35%, but again the glycine values are approximately the same as in the simple heterozygotes (H56).

5.3.5. *Some Genetic Considerations*

The wealth of information which indicates a linkage between the HPFH determinant and the Hb_β and Hb_δ structural loci [(B24, B54, F6, J3, K24, M1, S4, W6) and others] suggests linkage between the Hb_γ loci and the Hb_β and Hb_δ loci. Is, then, the HPFH condition caused by a physical deletion of Hb_β and Hb_δ and/or Hb_γ loci (H50, H52, H54, S24) or by a variation in a regulatory control mechanism which results in a failure to turn on the production of β and δ chains (M29, N11)? Because the data do not permit a definite conclusion, discussion of the genetic origin of the HPFH condition remains speculative. However, the striking heterogeneity of the anomaly (illustrated in Fig. 17) is probably best explained by assuming specific deletions of appropriate loci as well as by considering specific loci which control the relative production of β and δ chains and of $^G\gamma$ and $^A\gamma$ chains during the postnatal period. Because individuals in the Hb_{G_γ} and $Hb_{G_\gamma}Hb_{A_\gamma}$ classes produce no normal β and δ chains *in cis* to the HPFH determinants whereas heterozygotes of the Hb_{A_γ} class seem to synthesize β and δ chains *in cis*, an arrangement of loci in the manner $\delta\beta^A\gamma^G\gamma$ and deletions of specific loci will lead to the observed compositions of γ chains in the Hb-F. Data from analyses of γ chain variants suggest the presence of two Hb_{G_γ} and two Hb_{A_γ} loci and

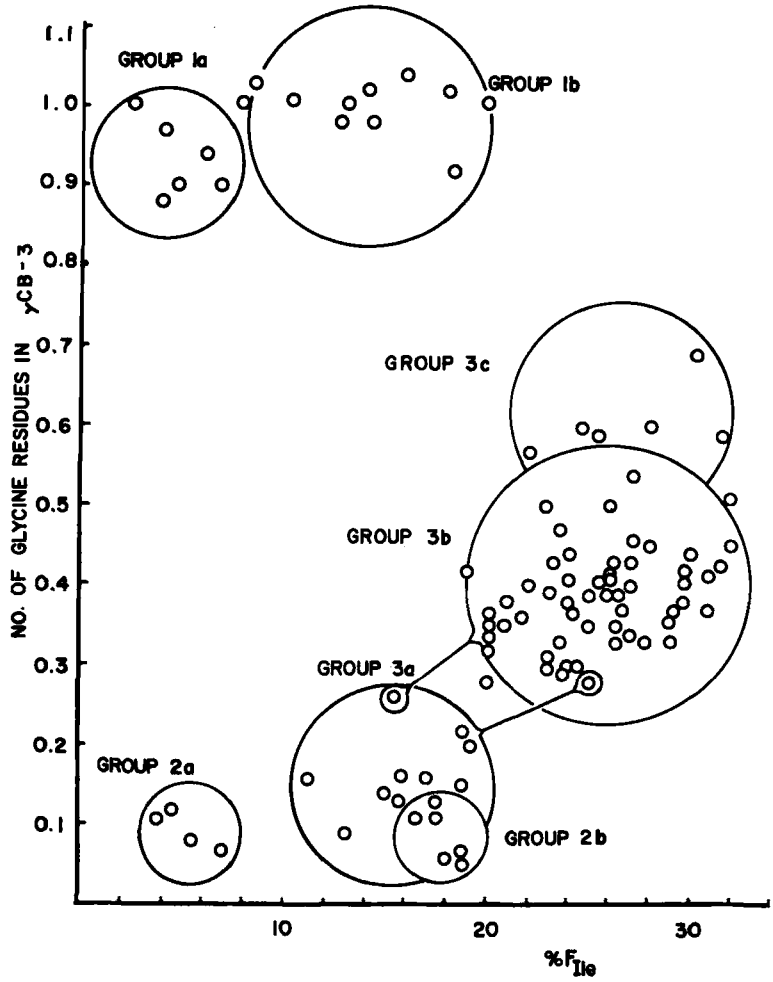

FIG. 17. The heterogeneity of fetal hemoglobin in subjects with a heterozygosity for HPFH. For further explanation see text and consult Tables 9–11.

deletions in arrangements which include these four loci may explain some of the additional subtypes of the HPFH conditions.

5.4. HETEROGENEITY OF HB-F IN THALASSEMIA

5.4.1. *β-Thalassemia* (S24, S25)

In this disorder the level of Hb-F is elevated in the homozygote and may or may not be increased in the heterozygote. The disease expresses

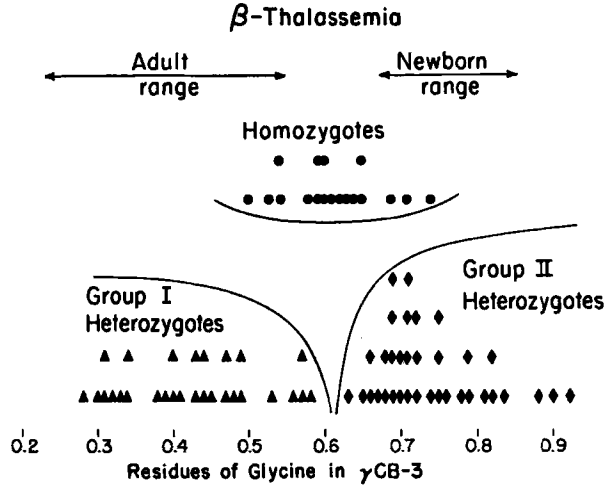

FIG. 18. The heterogeneity of fetal hemoglobin in β-thalassemia.

itself heterogeneously not only at the clinical, but also at the molecular, level because the production of β chains is variably suppressed (see Section 4). Chemical examination of the Hb-F in heterozygotes and homozygotes (i.e., patients with and without β chain production, and of different racial origin) separate the heterozygotes into two categories and the homozygotes into still another (Fig. 18). The two groups of heterozygotes fall in ranges that are characteristic of Hb-F of the adult (group I) and of the newborn (group II). This characteristic is an inherited trait because affected members of families fall either in group I or in group II.

The homozygotes form a group with intermediate values (mean value for glycine is 0.60); these homozygotes may be children of parents both of whom belong to group I or to group II, or one parent may belong to each group. This unusual relationship between homozygotes and heterozygous relatives is unexplained at present. The consistency of the data, however, indicates that the appearance of Hb-F with $^{G}\gamma$ and $^{A}\gamma$ chains in specific ratios is a primary manifestation of the condition. Perhaps the mechanism responsible for the change in the $^{G}\gamma$ to $^{A}\gamma$ ratio in the newborn infant after birth is affected.

5.4.2. *Hb-S(C)-β-Thalassemia*

Subjects with this condition usually produce larger amounts of Hb-F than do simple β-thalassemia heterozygotes. The ratio of $^{G}\gamma$ to $^{A}\gamma$ chains in the Hb-F is familially determined and will depend upon whether the family falls in group I or group II.

5.4.3. *HPFH–β-Thalassemia*

Subjects with HPFH (Hb_{A_γ} type; group 1b, Fig. 17) –β-thalassemia, and with HPFH ($Hb_{A_\gamma}Hb_{G_\gamma}$ type; group 3b; Fig. 17) –β-thalassemia have been studied (H54, H57). In both combinations, the production of Hb-F is considerably increased. Evidence from the glycine values of the Hb-F in these cases suggests that the increased synthesis of the γ chain is directed mainly by the β-thalassemia gene and not by the HPFH determinant.

5.4.4. *F-Thalassemia* (S61)

Heterozygosity for F-thalassemia is, among others, characterized by an increased amount of Hb-F (3.5–15% in 28 cases) and a normal level of Hb-A_2 (2.1–3.5% in 24 cases). Although detailed clinical and genetic studies of the condition have been made, the abnormality at the molecular level remains unknown (see Section 4). Chemical analyses of Hb-F from 27 heterozygotes have been made; in all cases both $^{G}\gamma$ and $^{A}\gamma$ chains were present in Hb-F in an average ratio of about 2:3 (mean glycine value 0.37 with a range of 0.25 to 0.56), which is similar to that found in the traces of Hb-F of normal adults. The increased synthesis of γ chains in this disease is apparently directed by both Hb_{G_γ} and Hb_{A_γ} loci and the regulatory factor that determines the ratio of $^{G}\gamma$ and $^{A}\gamma$ chains controls in favor of the "adult" ratio.

Homozygotes for this condition produce only Hb-F. In four homozygous subjects, the glycine values of 0.52, 0.49, 0.55, and 0.59 are significantly higher than those of the heterozygotes. In F-thalassemia also, as we have previously seen for HPFH ($Hb_{G_\gamma}Hb_{A_\gamma}$ type) and for β-thalassemia, the glycine values of homozygotes differ from those of heterozygotes. Analyses of Hb-F from four persons who have F-thalassemia in combination with β-thalassemia or with Hb-S gave data comparable to those of the F-thalassemia homozygotes; the percentages of Hb-F in these individuals is close to 90. It seems then that the molecular lesion in F-thalassemia completely suppresses of β and δ chain production *in cis* of the F-thalassemia determinant and increases production of all Hb_γ loci.

5.5. Heterogeneity of Hb-F in Some Hemoglobinopathies (H59)

Appreciable amounts of Hb-F can be present in patients with sickle cell anemia, with SC disease, with CC disease, with the Hb-Lepore disorders, and with hemolytic anemia due to the presence of an unstable hemoglobin variant [(A9, B21, B37, G6, H15, J1, L2, N9, R15, S11, S33, S49, T3) and others].

The level of Hb-F in SS patients may be as high as 20–30% in younger children (B37, S61) and in some patients of Arabic origin (A9, G6). There is some evidence that the level of Hb-F decreases with age during the first 20 years of life and is greater in older female patients than in male patients of a comparable age group.

Chemical analyses of Hb-F of AS, SS, and SC patients show a widespread ratio of the two types of chains (Fig. 19). It may be that a bimodal distribution is present, but the scatter in the data makes it unclear whether these patients fall in groups with glycine values of normal adults and newborns. Some indication of familial occurrence of these specific ratios may be evident.

From data on Hb-F of subjects with AC, CC, and the miscellaneous abnormalities of the rest group of Fig. 19 (A-Lepore, A-Sabine, A-Richmond, and heterozygotes for an unknown unstable variant) $^{G}\gamma$ and $^{A}\gamma$ chains are present in the nonspecific 2:3 ratio of the adult.

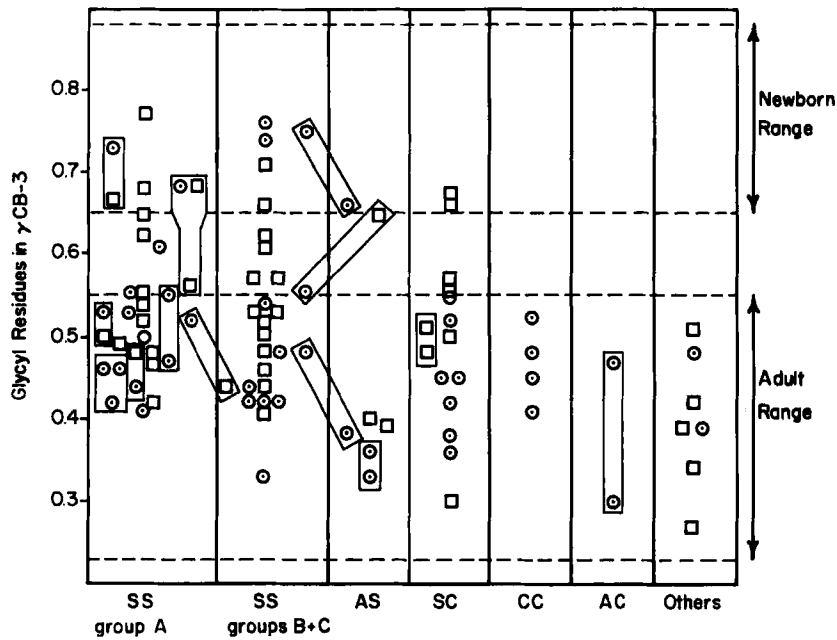

FIG. 19. The heterogeneity of fetal hemoglobin in subjects with various hemoglobinopathies. Group A represents SS patients of which the parents have been diagnosed as Hb-S heterozygotes; such information is incomplete for subjects of groups B + C. The group labeled "other" concerns patients with Hb-Richmond, Sabine, other unstable hemoglobins, and Hb-Lepore. Boxed-in values are from relatives.

5.6. Heterogeneity of Hb-F in Acquired Hematological Disorders

There are several acquired hematological disorders which have an increased synthesis of Hb-F [summarized in (H37, K13)]. Thus, an elevated level of Hb-F is frequently observed in, for example, Fanconi's anemia, megaloblastic anemia, polycythemia vera, various types of leukemia, multiple myeloma and lymphomas, macroglobulinemia, and metastatic disease of the bone marrow. The chemical heterogeneity of the Hb-F in some of these disorders (aplastic anemia, Fanconi's anemia, and various forms of leukemia) has been studied (H60, R29). In many instances, the $^{G}\gamma$ to $^{A}\gamma$ ratio is similar to that found in the normal newborn. This is particularly striking for patients with various forms of leukemia, and the Hb-F which is produced in increased quantities in these disorders invariably has a $^{G}\gamma$ to $^{A}\gamma$ ratio of about 3:1. This "newborn" ratio of $^{G}\gamma$ to $^{A}\gamma$ chains in Hb-F may be a common factor in these diseases; as perhaps another example of fetal characteristics in red cells of patients with leukemia, it supports the hypothesis that these cells may be of "fetal origin" (H25).

5.7. Heterogeneity of Hb-F in Nonhuman Primates

A heterogeneity of Hb-F similar to that of man has been found in the chimpanzee (J21). Chemical analyses have shown a great similarity between the primary structures of the γ chain of Hb-F of this animal species and that of the human. As in man, position 136 of the γ chain is occupied by a glycine residue as well as by an alanine residue. Apparently multiple Hb_{γ} structural loci are present in this species also. It is remarkable that the mechanisms which regulate the change in the $^{G}\gamma$ to $^{A}\gamma$ ratio between the third and fifth months after birth are also operative in the chimpanzee (J21).

More than one structural Hb_{γ} gene have also been observed in the *Macaca nemestrina* (N15, N16) but not in the *Macaca mulatta* and *Macaca speciosa* (J21).

6. Methodology

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Many techniques useful for the detection, isolation, quantitation, and characterization of hemoglobin abnormalities have recently been reviewed (C14, H37, J29). In this survey a limited number of methods will be discussed with major emphasis on new developments.

6.1. Hematology

6.1.1. *Heinz Bodies*

The presence of an unstable hemoglobin variant often leads to the occurrence of Heinz bodies in peripheral blood after incubation with a redox dye, particularly in a carrier who has been splenectomized. Three procedures are recommended:

a. A standard crystal violet solution in physiological saline is mixed with blood (freshly drawn in heparin) in a ratio of 2:1, and the mixture allowed to stand for 1 hour at room temperature. Heinz bodies are usually observed only when the carrier has been splenectomized (Fig. 20).

b. A standard solution of brilliant cresyl blue in physiological saline is mixed with blood in a ratio of 1:1, and the mixture incubated for 3 hours at 37°C. Heinz bodies can be found in carriers who have been splenectomized and also in those who have not.

c. Blood is incubated under sterile conditions at 37°C for 24 hours. After incubation, the technique mentioned in procedure a is applied. This method is (almost) always positive in cases where an unstable variant is present.

6.1.2. *Demonstration of Specific Hemoglobin Types within Erythrocytes*

An excellent survey which discusses these many microhistochemical techniques recently appeared (K14).

6.1.2.1. *Distribution of Hb-F and Hb-A in Erythrocytes.* The distribution of fetal hemoglobin within individual erythrocytes can be demonstrated by the technique of Kleihauer *et al.* (K17). Hemoglobin is precipitated inside the cells by drying and fixing the smears in ethanol. Precipitated Hb-A is more soluble in a citric acid–phosphate buffer, pH 3.3, than Hb-F perhaps because it dissociates more readily; thus, when the smears are placed in this solvent all Hb-A is eluted from the cells whereas most of the Hb-F remains precipitated. Staining the smears with specific dyes results in the cells with Hb-F being brightly stained and all others appearing as ghost cells.

Thin blood smears are made from fresh blood and allowed to dry in the air; the cells are fixed in 80% ethanol for 5 minutes. The slides are thoroughly washed with water and allowed to dry completely in the air, then they are placed in a McIlvaine citric acid–phosphate buffer, pH 3.3, at 37°C for 2–3 minutes (the time depends on the complete elution of the Hb-A from a control and should be determined experi-

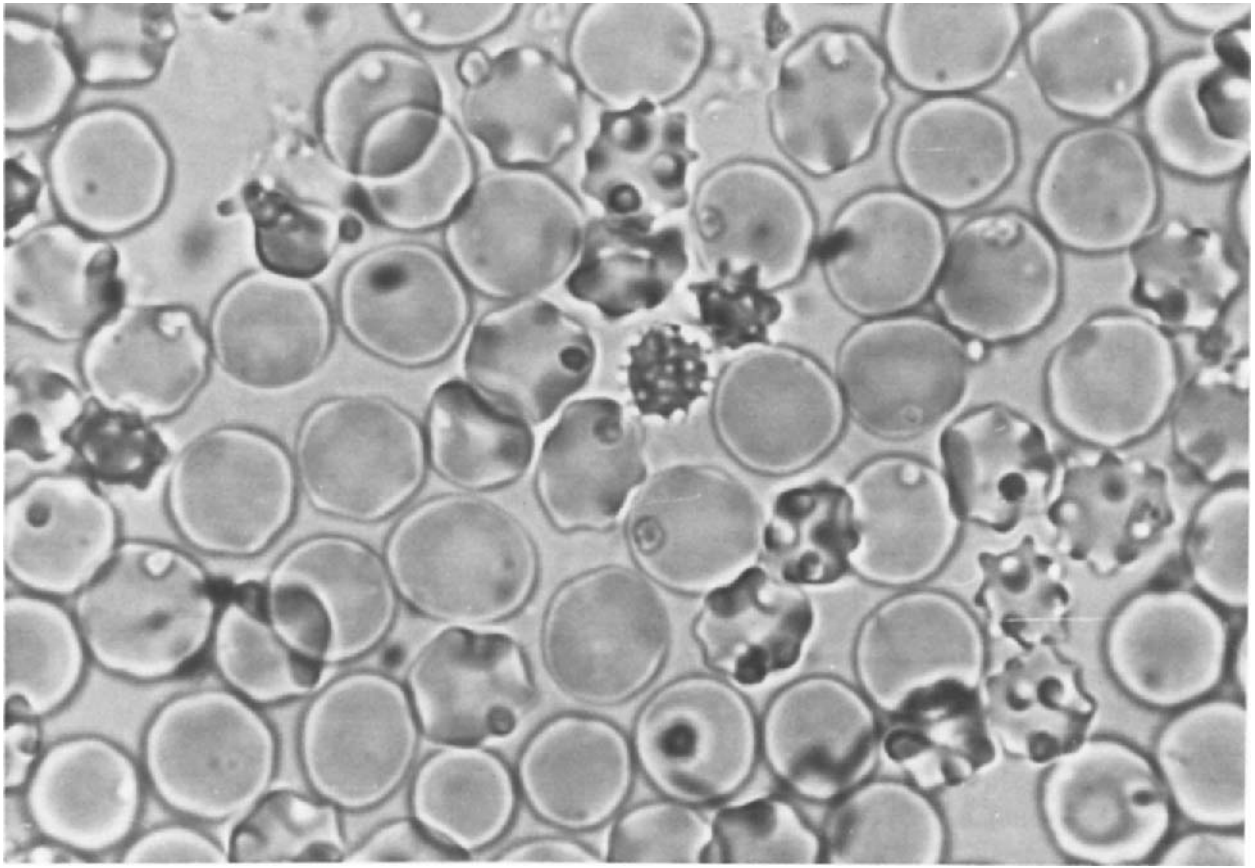

FIG. 20. The formation of Heinz bodies in a subject with the unstable hemoglobin Louisville. The patient was splenectomized at the age of 21 years. From Keeling *et al.* (K9) with permission of the authors and publisher.

mentally). The slides are washed with distilled water and stained in Erhlich's acid hematoxylin for 3–10 minutes, followed by a counterstaining in aqueous 0.1% erythrosin for 3–10 minutes. Cells with Hb-F appear pink while only a faint membranous outline is seen of other cells. The McIlvaine buffer is best prepared from a stock solution A (0.2 *M* Na_2HPO_4) and a stock solution B (0.1 *M* citric acid). Solution A is added to 75 ml of solution B until the desired pH is obtained (usually ±25 ml).

It may be that the staining of cells is not always due to the presence of Hb-F, and immature cells particularly stain with the dye because of the reticulum filaments that are present (B33). It is recommended to incubate the cells with a 1% brilliant cresyl blue solution in physiological saline for 4 minutes which will aggregate the RNA; satisfactory results can usually be obtained with this preparation and false positive F cells are often eliminated.

6.1.2.2. *Demonstration of Embryonic Hemoglobin in Erythrocytes.* This method is a slight modification of the previous procedure and requires that the blood be incubated for 20 minutes in a humid atmosphere with an equal part of a 1% solution of brilliant cresyl blue in Ringer's solution. The slides are prepared from these incubated cells and are allowed to dry for at least 2 hours in the air. The dried slides are fixed in 96% ethanol for 5 minutes, rinsed with water, and again dried completely in air. The elution requires a citric acid–phosphate buffer, pH 3.4–3.5. The staining procedure is the same as described in the previous procedure. It is also possible to incubate the cells first with a 1% brilliant cresyl blue solution in physiological saline, followed by fixation in 80% ethanol. The pH of the McIlvaine buffer solution should then be lowered to 2.9. With both procedures adult and fetal hemoglobins are eluted from the red blood cells and the embryonic hemoglobin remains inside as a precipitate. Staining with erythrosin identifies the cells which contain embryonic hemoglobin, and the reticulate core in fully eluted erythrocytes can easily be identified. The reader is referred to reference K14 for further details.

Methods have also been developed for the demonstration of Hb-S within the red cell, and for that of ferrihemoglobin and CO-hemoglobin; the reader is referred to (K14) and to references quoted in that review.

6.2. Electrophoresis

Electrophoretic methods using supporting media, such as starch, starch-gel, cellulose acetate, agar gel, continue to be of importance in detecting abnormal hemoglobin variants. The techniques are less suit-

able for the identification of abnormal hemoglobins because many variants have identical electrophoretic mobilities. Some new developments concern the electrophoretic separation of hemoglobin from single red blood cells, and the isoelectric focusing of hemoglobins.

6.2.1. *Electrophoresis of Hemoglobin in Single Erythrocytes*

All methods used for identification of hemoglobin types depend upon a massive number of cells. Matioli and co-workers (M9, M10, M11, M12) have been successful in the separation of Hb-F and Hb-A from a single cell. The method uses polyacrylamide gel as a supporting medium. Separation of Hb-A from Hb-F and also from Hb-A_2, Hb-S, Hb-C, and Hb-J has been made. An interesting modification has recently been described (D2).

6.2.2. *Isoelectric Focusing of Hemoglobin Components*

Successful application of this method depends upon the difference of at least 0.02 pH unit in the isoelectric points of the hemoglobins undergoing electrophoresis. Linear pH gradients are attained in 5% polyacrylamide gels and the passage of an electric current through the gel causes the hemoglobins to migrate to their isoelectric points producing sharp narrow bands. Quantitation can be made either with the use of a densitometer or by elution of each band. The technique claims to resolve minor hemoglobins which are difficult to separate by other methods (B74, B78, D11). A short description of the method of Drysdale *et al.* (D11) for human and animal hemoglobin is presented here.

Solutions of 4% acrylamide and 2% ampholyte are polymerized for gel formation by the addition of *N,N,N′,N′*-tetramethylethylenediamine (0.02%) and ammonium persulfate (0.02%). The solution after degassing is poured into 10 × 0.3 cm tubes which are sealed at one end with dialysis membrane tubing. Overlaying the gel with water will produce a flat, even surface.

An apparatus similar to that used in the popular disc electrophoretic technique can be used for isoelectric focusing. One modification concerns the insertion of the tubes in a jacket through which water of 2°C can circulate; only the tips of the tubes will be in contact with the electrolytes. Platinum electrodes should be used and arranged at the extremities of the tubes. The anolyte and catholyte are 0.02 *M* phosphoric acid (pH 2.2) and 0.01 *M* sodium hydroxide (pH 12.0). Excess persulfate should be eliminated by maintaining a current of 1 mA per tube for 10 minutes.

Between 10 and 30 μl (200 μg of hemoglobin), dissolved in 4%

ampholyte, is applied to the top of the gel. A current of 1 mA per tube at 500 V is maintained until separation occurs and the isoelectric points are reached. The gels may be scanned at 576 nm.

6.3. Column Chromatography of Hemoglobin

Ion-exchange resins such as DEAE-Sephadex, CM-Sephadex, Amberlite IRC-50, DEAE-cellulose, and CM-cellulose have been introduced and methods for their use in hemoglobin separation have been developed by many investigators [summarized in (H37, J29)]. CM-Cellulose particularly has proved to be a highly versatile resin. With this weak cation exchange resin and phosphate buffers of low ionic strength and an increasing pH gradient excellent separation of even minor hemoglobin components is possible. Stock solutions A (0.01 *M* Na_2HPO_4) and B (0.01 *M* NaH_2PO_4) are used for making all other developers which also contain 100 mg of KCN per 1000 ml. Conditions for the development of a chromatogram are dependent on the problem involved and an initial trial elution pattern is often necessary. The most useful CM-cellulose is CM 52 (microgranular, preswollen, with a capacity of 1.0 mEq/g dry; Reeve Angel, Clifton, N. J.). This resin requires no prior treatment except equilibration with the starting developer for at least 48 hours.

A column (1.5 × 35 cm) is prepared using the equilibrated CM 52 cellulose. Approximately 40 mg of hemoglobin (dialyzed for 24 hours against the starting developer) is applied to the top of the resin. A pH gradient is established by using a 250-ml mixing flask with the starting developer; the mixer is connected to a 500-ml funnel containing the limiting developer. A flow rate of 4 ml per 20 minutes is recommended. During chromatography the limiting developer is changed to one with a higher pH, thus creating a smooth pH gradient. The pH of every tenth tube is recorded, and the extinction of every tube is read at 415 nm. The quantitation of each fraction can be calculated from the absorbency. The procedure is adaptable for preparative chromatography. Figure 21 shows some examples of separations possible with the procedure.

6.4. Quantitation of Fetal Hemoglobin by Amino Acid Analysis

For many years, alkali denaturation has been the most popular method for the quantitation of Hb-F in human red cell hemolysates. However, these denaturation techniques have always been subject to question because of interfering properties in the procedure. Chromatographic determination of Hb-F, while providing a separation of the fetal component from the major Hb types, is still subject to error because minor adult hemoglobins may elute together with Hb-F.

A method has been devised which uses chromatographic procedures

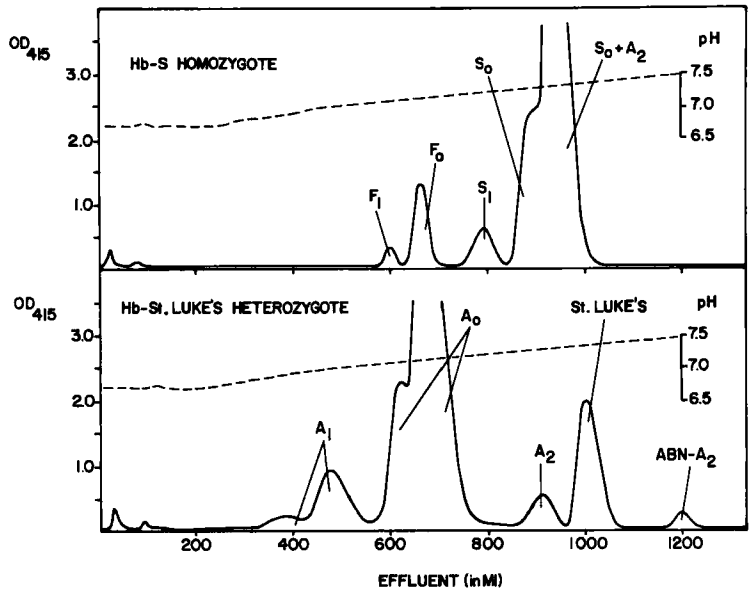

FIG. 21. Separation of hemoglobin components by chromatography on a column of CM-cellulose. Red cell hemolysates from a 19-year-old female patient with sickle cell anemia (top graph), and from a subject with a heterozygosity for the α chain variant Hb-St. Luke's (bottom graph).

in combination with amino acid analysis (S26). The $\alpha\gamma$ dimer of Hb-F contains four residues of isoleucine, 35 residues of leucine, and 15 residues of phenylalanine. The $\alpha\beta$ dimer of Hb-A contains no isoleucine residues, 36 residues of leucine, and 15 residues of phenylalanine. The determination of the content of isoleucine in relation to leucine and phenylalanine can therefore be used as a measure of Hb-F in mixtures. This procedure involves the chromatographic isolation of the fetal hemoglobin containing zone and the determination of its isoleucine, leucine, and phenylalanine content by amino acid analysis. The method is not suitable as a routine procedure.

Approximately 40 mg of hemoglobin is applied to a 0.9×45 cm column of DEAE-Sephadex and the chromatogram is developed with a gradient of 0.05 *M* Tris·HCl buffers (D8). Fractions containing Hb-F are pooled, the pooled effluent deluted once with water, and one or two drops of a 2% KCN solution are added. The pH of this solution is adjusted to 6.5–6.7 with 1.0 *M* maleic acid. CM-Sephadex (C-50, capacity 4.5 ± 0.5 mEq/g, particle size 40–120 μ, Pharmacia Fine Chemicals) which is equilibrated with 0.05 *M* Tris·maleic acid buffer, pH 6.5, is used to prepare a column of 0.5×2.0 cm for concentrating the hemo-

globin component. The hemoglobin will accumulate at the top of the resin and can be eluted completely with a few milliliters of 0.05 *M* Tris·HCl buffer pH 8.2–8.5 containing 100 mg of KCN per liter.

The eluate from the CM-Sephadex column is next dialyzed against distilled water at 4°C for 12–24 hours. A small quantity of protein is hydrolyzed in 6 *N* HCl for 72 hours at 100°C. After hydrolysis the acid is removed in a stream of filtered air and an appropriate aliquot of the hydrolyzate taken for analysis of isoleucine, leucine, and phenylalanine, an automatic amino acid analyzer being used. The percentage of Hb-F in the sample is calculated using this formula:

$$\%F = \frac{\mu\text{mole Ile} \times 100}{2\left(\frac{\mu\text{mole Leu}}{35.5} + \frac{\mu\text{mole Phe}}{15}\right)}$$

Accuracy of the method is in the range of about 5%.

6.5. Detection of Unstable Hemoglobin Variants

It has been shown that the stability of the heme group is one of the most important factors in the denaturation (and precipitation) of hemoglobin both *in vivo* and *in vitro* (R17). Thus, when the replacement of an amino acid residue in the chain occurs in a position which is part of the heme contact, the stability of the molecule is usually affected, resulting in loss of the heme group from that particular chain and in a progressive denaturation. Rapid denaturation, therefore, is a characteristic of an unstable hemoglobin (R20). Precipitation of the protein takes place within the cell and intraerythrocytic inclusion bodies appear leading to increased cell destruction.

6.5.1. *Denaturation by Heat*

This rapid denaturation of hemoglobin affords this method for determining the presence of an unstable hemoglobin variant even when starch gel electrophoresis of hemolysate shows no evidence of an abnormality. The heat stability of the hemoglobin variant is compared with that of a normal control; the two are incubated simultaneously in a phosphate buffer at 60°C (K9).

One to 2 ml of a freshly prepared red cell hemolysate is diluted to 50 ml in a final concentration of 1.5–2.0 mg/ml. Exactly 4.5 ml of hemoglobin solution is pipetted in test tubes labeled 0, 2, 4, 6, 8, 10, 15, 20, 30 minutes. Of a 2.5 *M* potassium phosphate buffer (pH 6.9), 0.5 ml is added to each tube. After mixing, the tubes are placed in a 60°C water bath. The tubes are removed from the water bath at the times indicated and placed immediately in an ice bath for 5 minutes. The

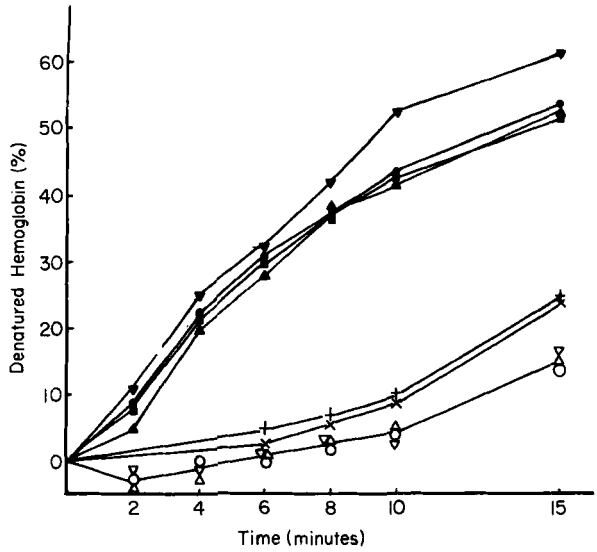

FIG. 22. Heat denaturation curves of hemoglobin from five normal subjects (○—○, A.E.; △—△, C.E.; ▽—▽, K.E.; +—+, M.E.; ×—×, J.E.) and four patients with Hb-Louisville (●—●, He.E.; ■—■, F.E.; ▲—▲, H.E.; ▼—▼, Jo.E.). Temperature 65°; pH 6.9. From Keeling *et al.* (K9) with permission of the authors and publisher.

precipitate can be removed by filtration or by centrifugation. The extinction of the supernatant is read at 523 nm, which is the approximate isobestic point for oxy-, deoxy-, and ferrihemoglobin. Figure 22 illustrates some denaturation curves.

6.5.2. *Separation of the Hemoglobin Molecule into Its Constituent Subunits*

A popular method is the procedure developed by Bucci and Fronticelli (B75) using *p*-chloromercuribenzoate (PCMB); the method allows a high yield of native subunits of many hemoglobins. Modifications of this method have broadened its applicability to hemoglobin variants that are more difficult to dissociate (D4, E5, K1, R31, T14). It has been shown that the reaction sites of the hemoglobin molecule are the cysteine residues at β 112 and β 93 (S34). It is necessary that both sulfhydryl groups be present, and less dissociation of the molecule occurs when one of these groups is missing (as in Hb-F).

The following modification of the procedure of Rosemeyer and Huehns (R31) has been used in this laboratory. Reagents: 0.1 *M* sodium phos-

phate buffer; 2 *M* NaCl; 1 *N* NaOH; PCMB solution. This solution is prepared by dissolving 36 mg of PCMB in 0.8 ml of distilled water and 1 ml of 1 *N* NaOH. The solution is titrated with 1 *N* acetic acid until a slight turbidity (at about pH 10) whereafter the volume is adjusted to 3.6 ml with distilled water. Prepare fresh just before use. CO-hemoglobin is used, which is prepared by passing a stream of CO gas over a red cell hemolysate for about 5 minutes or until a deep color appears.

The reaction mixture is prepared in the following ratio: 1.0 ml of CO-hemolysate, 5.0 ml of phosphate buffer, 1.0 ml of 2 *M* NaCl, 2.0 ml of distilled water, and 1.0 ml of PCMB solution, which is added last. The mixture is stirred for 1–24 hours (depending on the amount of precipitate that is produced) at 4°C under CO tension. The precipitate is isolated by centrifugation and washed with a solution prepared in this ratio: 10.0 ml phosphate buffer, 2.0 ml 2 *M* NaCl, and 4.0 ml distilled water. The PMB may be removed from the protein by dissolving the precipitate in a 2% KCN solution and dialyzing the solution against water for 24 hours.

An easy method for the detection of abnormal α or β chains in hemoglobin variants is based on the observation that treatment of the hemoglobin with *p*-hydroxymercuribenzoate (PMB) produces monomers and dimers that can be separated by electrophoresis. Each test requires four small test tubes: a control tube and a reaction tube for each of two molar concentrations of PMB.

The concentrations are: 4 moles per mole of hemoglobin, which corresponds to 15 mg of PMB in 10 ml of 0.05 *M* Tris·HCl buffer, pH 7.5; and 12 moles per mole of hemoglobin, which corresponds to about 45 mg per 10 ml of Tris buffer. These solutions are mixed with an equal volume of red cell hemolysate (6.5 g of Hb per 100 ml); the reaction is allowed to take place for at least 4 hours at room temperature. Identification of subunits can be made by starch gel electrophoresis at pH 8.6 or 9.0. Separation of the subunits is enhanced if PCMB is added to the buffer vessels. Figure 23 illustrates the method.

6.6. Radioactive Amino Acid Incorporation in Hemoglobin

Some phases of the synthesis of hemoglobin have been studied *in vitro* and *in vivo* in both animal and human subjects by various investigators [(G9, H11, M4, N13) and others]. Labeled amino acids, i.e., leucine-^{14}C, phenylalanine-^{14}C, and leucine-^{3}H, are used for the study of amino acid incorporation. The procedure consists of obtaining either human or animal mRNA, reticulocyte ribosomes from the study case, and radioactive amino acids. The three are combined and incubated for

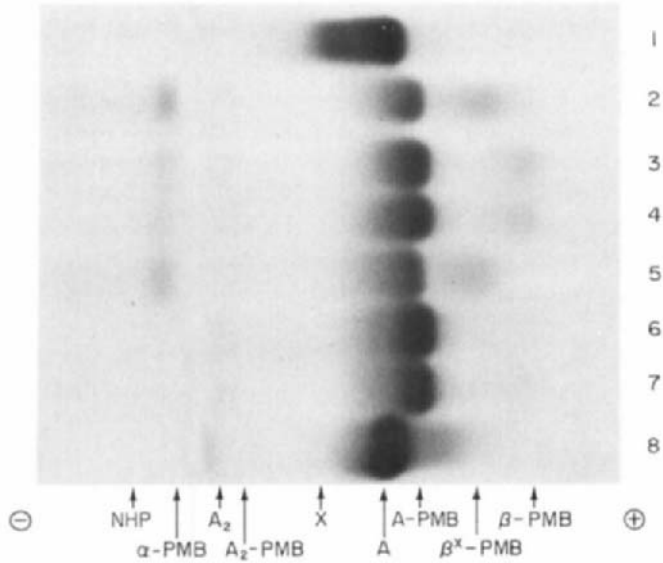

FIG. 23. Starch gel electrophoresis at pH 9.0 and 4°C of hemolysates treated with PMB. Samples 1 and 8, untreated hemoglobin from subject with the unstable Hb-Savannah and her normal father, respectively; samples 2 (propositus), 3 (normal mother), and 4 (normal father) were treated with 12 moles of PMB per mole of hemoglobin; samples 5 (propositus), 6 (mother), and 7 (father) were treated with 4 moles of PMB per mole of hemoglobin. Gel is stained with Amino Black 10B. NHP denotes nonhemoglobin protein fraction, X the abnormal hemoglobin. From Huisman *et al.* (H55) with permission of the authors and publisher.

a specified time. Radioactivity of the hemoglobin or of each of the two types of chains is determined by scintillation counting.

The isolation of the individual chains is usually made with a chromatographic method pioneered by Clegg *et al.* (C18). Modifications of the method have been made to suit specific purposes in the isolation of the chains of both animal and human hemoglobin. Type of resin, size of column, pH, and molarity of developers have been adjusted or changed for development of the chromatogram desired (A2).

The following modification is at present in use in the author's laboratory: Thirty grams of resin (CM-52 preswollen) is equilibrated in developer A (0.005 *M* sodium phosphate, pH 7.0, in 8 *M* urea and 0.05 *M* β-mercaptoethanol) for three changes; a column (2.0 × 12.0 cm) is then poured. Approximately 200 mg of globin is dissolved in a few milliliters of developer A and dialyzed against the same solution for 60–120 minutes. The protein is applied to the top of the column, and the chromatogram is developed with a descending flow of solvent. Two cylinders

equipped at the bottom with stopcocks are used to develop an increasing molarity gradient. Developer A, 400 ml, is placed in the cylinder, which is connected to the column. The second cylinder contains 400 ml of developer B (0.04 *M* sodium phosphate, pH 7.0, in 8 *M* urea with 0.05 *M* β-mercaptoethanol). This cylinder is joined to the cylinder containing developer A, which serves as the mixing vessel. The flow rate is maintained at 1 ml per minute. Figure 12, Section 4, illustrates some separations obtained with this procedure. The use of hemoglobin instead of globin may have distinct advantages.

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THE ENDOCRINE RESPONSE TO TRAUMA

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1. Introduction

A surgical operation or any form of physical injury in a previously healthy person initiates a series of metabolic changes in which protein tissue is catabolized, fat is oxidized, and water and salt are retained (B10, C13). The endocrine system responds at the same time with an increased secretion rate and altered tissue utilization of many hor-

mones. The endocrine and metabolic responses are closely interrelated and cannot easily be considered as separate subjects. Increased production of hormones after injury is part of a basic defense mechanism for survival, and absence or failure of some glands, such as the adrenal cortex, is associated with circulatory collapse after injury.

The mechanism by which the endocrine and metabolic response is activated has not been defined clearly. There is evidence that both neural impulses and tissue substances play a part in stimulating what is virtually a total increase in endocrine activity. Many of the hormones which are increased have metabolic functions which include the maintenance of blood glucose levels. This appears to be a fundamental biological defense mechanism whereby an essential energy source is preserved for the central nervous system.

While it is convenient to consider each endocrine gland separately, the functional interrelationships between endocrine and metabolic changes must be kept in mind.

2. Adrenocortical Secretion

The most striking aspect of the endocrine response involves the adrenal cortex. Albright was among the first to observe a raised output of urinary steroids in injured patients and to note the similarity between the postoperative response and the changes of Cushing's syndrome (A2).

2.1. Cortisol

The development of the Nelson and Samuel's method enabled free 17-hydroxycorticoids to be measured in the plasma. There are many reports describing a rapid rise in the plasma cortisol levels during a surgical procedure. Peak values about five times the basal levels are reached within 5 or 6 hours and then the level returns to normal within the next 12 hours (B2, Y1). The plasma cortisol level in the blood at any time depends on the balance between the rate of secretion and the rate of conjugation and excretion (F2). The rate of removal of cortisol from the plasma is related to hepatic blood flow which is depressed during major surgery or immediately after injury so that a reduction in hepatic conjugation of cortisol may contribute to the high blood levels (T4).

Studies involving standard infusions of cortisol to patients before and immediately after surgery indicate that the rate of disappearance of 17-hydroxycorticosteroids is reduced by stress (M1), and it would appear that the impaired hepatic removal of free 17-hydroxycorticosteroids is an important factor in maintaining raised plasma levels

immediately after surgery (T4). The output of cortisol from the adrenal has been measured in the adrenal vein during operation and a 10-fold increase from 3.5 mg per minute to 30–50 mg per minute has been recorded (H12). Rapid methods for measuring plasma cortisol are now available, and the adrenocortical response to injury can be assessed by measuring plasma cortisol at regular intervals during and after operation (M5). The excretion of cortisol and its metabolites in the urine is raised for a much longer period than that during which the plasma cortisol is raised and probably gives a more complete picture of the total adrenocortical response (Y2). Cortisol metabolites usually measured as 17-hydroxycorticoids are increased for 3–4 days after surgery of moderate severity (M8, P1) rising from 3–5 mg/day to about 18–20 mg/day within 3 days (M8). The extent and duration of the increased urinary excretion of cortisol metabolites depends upon the severity of the injury and complications such as secondary hemorrhage or sepsis prolong the period of increased output (M9). The adrenal has a very great capacity to secrete at a high rate for long periods of time.

The output of 17-oxosteroids and metabolites of adrenal androgens barely alters after injury (H12). The results of a detailed study and a review showed that the oxosteroids showed no change as a result of a surgical operation (M8). A recent study in which the urinary 17-oxosteroids were measured using mild hydrolysis of glucuronide and sulfate and purification with elution chromatography showed a significant decrease in the excretion of individual 17-oxosteroids for 2–7 days after surgery, particularly in males (T1).

The usefulness of plasma cortisol measurements and urinary steroid levels is restricted because of the rapid fluctuations in hormone levels in blood and the possible important variations within different groups of urinary steroids which cannot be detected when only total excretion is measured.

Cortisol in plasma is present either in a bound or free form.

The free component of plasma cortisol is biologically active and is filtered and excreted in the urine, where it can be measured. It would appear that urinary free cortisol estimations may give a more accurate picture of glucocorticoid activity at a tissue level than most other measurements.

Espiner found that the 24-hour urinary free cortisol excretion ranged from 20 to 320 μg (E6). Four out of fourteen patients had levels in excess of patients with medical stress and comparable to levels found in patients with florid Cushing's syndrome. Similar observations have been recorded in other preoperative patients (F3). It is suggested that these levels are due to anxiety about impending surgery even though

the patients with high levels showed no outward signs of fear. Emotion is a known potent stimulus for adrenal secretion in man (H8, H10, H11).

Major surgical procedures produced the greatest increase in urinary free cortisol. Abdominoperineal resection of the rectum led to a 30-fold increase in urinary free cortisol. The response after ligation and stripping of varicose veins was comparable to major stress, indicating that the response may be related to the amount of tissue damage and afferent nerve stimulation. However, not every operation produces a response. About 20% of patients undergoing minor procedures showed no change in urinary free cortisol (E6, T1).

The response during any major surgical procedure is maximal because the administration of adrenocorticotropic hormone (ACTH) during major surgery produces no further increase in the plasma cortisol level, but within 24 hours there is an immediate response to a further ACTH stimulus.

There is some evidence that the responsiveness of the adrenal cortex to stress is related to blood flow through the gland. Patients in severe shock have been found to have very low plasma cortisol levels, which rise sharply after successful resuscitation (F2). Experimental studies show that cortisol production rose promptly with modest hemorrhage, but fell rapidly when shock was produced (M1). There is also evidence that in severe hypovolemic states the blood flow is preferentially shunted from the adrenal cortex to the adrenal medulla (M1). Interpretation of plasma cortisol levels in shocked patients is thus difficult without some idea of adrenal perfusion.

2.2. Aldosterone

The potent sodium-retaining property of this hormone is one explanation of the sodium retention and potassium excretion in the postinjury period (M2). Early evidence of aldosterone activity after injury was detected in adrenalectomized rats by observing changes in sodium potassium levels in the rat urine following injection of urine samples (L1). With these methods, increased aldosterone-like activity could be detected in postoperative urine. The urinary electrolyte changes could be due to increased cortisol levels as well. Further indirect evidence of the role of aldosterone in postoperative electrolyte changes comes from studies using the aldosterone blocking agent spironolactone.

The mean fall in sodium potassium ratio was similar on the first postoperative day in patients in both control and spironolactone-treated groups; this finding suggested that at that time factors other than aldosterone were controlling sodium and potassium losses. However, in subsequent days more sodium was lost, and potassium was retained, in

the group giving the blocking agent (J2). The displacements of sodium after trauma are only partially related to aldosterone (K1). Adrenalectomy patients on constant steroid replacement have normal sodium retention after injury.

Direct estimation of aldosterone in blood and urine shows a marked rise after injury (Z1). The secretion rate can be measured with a double isotope dilution technique, and increased amounts have been found in the adrenal vein blood and in the urine (D1, U1). The pattern of aldosterone release follows that of cortisol and is related in time and extent to changes in sodium and potassium balance and the severity of the injury (C5).

2.3. Renin and Control of Aldosterone Release

Aldosterone secretion is under the control of the renin-angiotensin system and peripheral plasma renin levels have been found raised during laparotomy and in response to hemorrhage (C10).

It was concluded from these and other studies in hypophysectomized animals that ACTH had no effect on aldosterone release. The volume-sensitive renal mechanism appears to be mainly responsible for post-operative aldosterone changes (S4), but it would now appear that ACTH also plays a part in regulating aldosterone secretion (S4). Removal of the pituitary leads to an immediate fall in aldosterone levels in adrenal venous blood (H9). A linear dose response relationship exists between the infusion rate of ACTH and aldosterone secretion rates (H9). Volume receptors in the right atrium and in the vascular tree respond to minor reductions in blood volume and play an important part in stimulating the aldosterone response (B1, F1). Patients with suppression of cortisol production due to prolonged administration of steroids continue to secrete aldosterone and are able to increase their output after stress indicating the presence of another trophic factor as well as ACTH (T3).

The maintenance of blood volume after open heart operations by accurate measurement of losses with immediate replacement led to a reduction in aldosterone secretion compared to operations where careful monitoring of changes in blood volume was not practised (W2). The concentration of plasma cortisol behaves in a similar fashion during open heart surgery and significantly lower levels are recorded than during major thoracic operations (C7, S4). These studies emphasize the importance of volume receptors in the right atrium and juxtaglomerular body in the kidney and emphasizes the importance of maintaining vascular homeostasis during and after surgery (S5).

It has been suggested that in contrast to adult patients infants have

a postoperative diuresis and sodium is lost instead of being retained (C9). The aldosterone secretion rate was measured in three groups of infants using a double isotope dilution technique. When the surgical stimulus was minor, like a herniotomy, no increase was detected but with more major procedures all infants showed an increase in secretion rate.

The mean value of the aldosterone secretion rate in newborn infants is significantly less than in older infants (W4). Careful monitoring of sodium and potassium balance is required in the neonatal period because of a potential insufficient secretion of aldosterone.

Many of the components of injury can influence aldosterone secretion, and sodium depletion and hypovolemia are probably the most important (B7, S5). Hemorrhage alone can also stimulate aldosterone secretion rate. A raised concentration of potassium in blood and excess 5-hydroxytryptamine from injured tissues have both been shown to increase aldosterone secretion (M11).

There are thus at least three mechanisms associated with injury, ACTH, blood volume changes, and the release of cellular potassium, all of which can mobilize aldosterone. ACTH mobilization can occur in the absence of volume changes or an excessive leakage of cellular potassium, so it probably has the major role to play. Evidence for this comes from a study in a hypophysectomized subject undergoing surgery in whom blood volume changes and tissue damage were minimal. No changes in urinary aldosterone were recorded in this patient (Z1).

2.4. Permissive Role of the Adrenal Cortex

The metabolic effects of exogenous cortisone are similar to the sequence of events following injury. A close correlation was found between urinary cortisol excretion and nitrogen losses in surgical patients (M10). For these reasons it was considered that the adrenocortical hormones initiated and controlled the metabolic response to injury. There are serious objections, however, to this hypothesis, as the complete interdependence of the responses has been demonstrated in man and animals.

After major operations the adrenocortical response as measured by urinary 17-hydroxycorticoid secretion is completed many days before metabolism returns to normal.

The metabolic response in patients undergoing total hypophysectomy or adrenalectomy (and in those undergoing surgical procedures some time after these endocrine ablation procedures) follows the characteristic course provided constant maintenance doses of cortisol are given before and after surgery. Increases above the maintenance doses are not required (J5). Constant replacement therapy does not ensure a constant

blood cortisol level after injury due to alterations in hepatic conjugation. Patients who are severely underweight and ill at the time of surgery do not exhibit a significant metabolic response but have normal high levels of cortisol in blood and urine after surgery similar to those found in well nourished patients (J6).

The catabolic response of patients after surgical operations as measured by nitrogen balance can be diminished significantly by giving anabolic steroids without affecting in any way the adrenocortical responses (J1).

Experimental studies in rats indicate that the adrenal response as measured by plasma cortisol levels is unaltered by keeping injured animals at 30°C, though this temperature reduces significantly the catabolic response (C1, T5).

Numerous studies have been carried out in animals in which total adrenalectomy has been performed and subsequent injury has not been accompanied by replacement therapy. The α_2 acute phase globulins rise after repeated injurious stimuli in rats; adrenalectomized animals retain the α_2 globulin response and serum mucoid response to tissue damage although it is reduced. Replacement therapy with cortisol in adrenalectomized animals restores the response to normal (W3). Adrenalectomy suppresses the breakdown of liver polypeptides and urinary nitrogen excretion in rats subjected to whole-body irradiation (N1).

The effects of injury and corticoid administration on protein metabolism differ significantly in animals. The content of liver nitrogen is increased by giving cortisone to rats but fracture of the femur does not have this effect in spite of increased levels of cortisol in the blood (M13). The administration of cortisone has a constant effect on nitrogen balance at all levels of nitrogen intake whereas the catabolic response to injury is reduced or even abolished by diminished protein intake and weight loss prior to injury (M12).

All these studies indicate that the metabolic response to injury cannot be explained completely in terms of increased adrenocortical activity and confirm the hypothesis of Ingle (I1) that the secretions of the adrenal cortex play a permissive rather than a causative role in postoperative metabolic changes. In other words, the presence of the adrenocortical secretions is necessary for the metabolic response to occur, but the secretions do not themselves initiate the response.

3. Anterior Pituitary

The anterior pituitary response to injury has now been examined in detail by means of radioimmunoassays.

3.1. Adrenocorticotropic Hormone

Most anterior pituitary hormones are secreted in response to a negative feedback mechanism whereby increases in the secretions of the target glands inhibit further pituitary hormone production. The hypothalamus is important in the negative feedback chain as it synthesizes link or releasing hormones which reach the anterior pituitary by the hypothalamohypophysial portal system to stimulate hormone secretion. There is no information yet of the effect of injury on most of the releasing hormones of the hypothalamus. The anterior pituitary is an important relay center in the response to injury. Destruction of the median eminence in experimental animals prevents the release of ACTH in response to stress (H11).

The pituitary glands of patients dying very soon after severe injury show the absence of granules in basophile cells, and this has been taken as evidence of a sudden increase in release of ACTH following trauma (C10). There is a sudden increase in the level of ACTH in blood following trauma with a fall to normal levels within a few hours (C10). ACTH plays an important part in the secretion of cortisol after injury and the amount of ACTH in the circulation is greater than that required for a maximal adrenocortical response. The effect of injections of ACTH has been compared with the response to injury, and an interesting difference emerges. The administration of standard ACTH infusions on either the first or second postoperative day produces higher plasma cortisol levels than in the preoperative period. This adrenal responsiveness may persist for as long as 10 days after injury (S4). ACTH increase the excretion of both 17-oxogenic steroids (metabolites of cortisol) and 17-oxosteroids (androgen metabolites) whereas surgical operation stimulates the release of the former. It may be that stress influences adrenal steroid biosynthesis by means other than ACTH stimulation and that stress changes the metabolism of androgen (J2). It may be of course that androgen secretion from the testes is so reduced following injury (M3) that any increase due to ACTH is masked (T1).

ACTH is also involved in stimulating the secretion of aldosterone after surgery. The secretion of both cortisol and aldosterone are related directly to the secretion rate of ACTH but aldosterone is also influenced by other factors (Section 2.2).

Under normal conditions the secretion of ACTH by the pituitary is related to the plasma cortisol concentration. A rise in plasma cortisol reduces the ACTH output of the pituitary. During surgical procedures the regulation of ACTH secretion is altered and levels of plasma cortisol which normally suppress ACTH output no longer do so and plasma ACTH levels rise. During surgical procedures the concentration of

ACTH in plasma of patients receiving exogenous cortisol was similar to that in patients undergoing similar operations and not given cortisol (E7). Large doses of corticosteroids have a restraining influence on the pituitary response to some minor surgical procedures (M1). There is considerable evidence, however, that the regulation of ACTH secretion during stress is outside the control of the normal negative feedback mechanism. It has been suggested that the negative feedback system does persist during stress but that the control point is raised. There is no obvious evidence of any control even at a higher set point so that no feedback control probably occurs after injury (Y1).

3.2. Growth Hormone

The observation that the pituitary glands of patients dying within a few days of major trauma contained very small amounts of growth hormone (G3) was interpreted as evidence of increased secretion during stress. Human growth hormone secretion can be stimulated by fear, emotion, and pyrogens as well as tissue damage (G5). Exercise will increase the circulating growth hormone level, and competitive sport is a more potent stimulus than exercise alone. Hemorrhage was found to be a most potent stimulus of growth hormone secretion in monkeys, but this finding was not confirmed (K2).

Growth hormone concentrations increase rapidly from 2.0 to 16.7 μg/ml by the first hour of a major operation. The levels then fall later in the procedure to around 6.7 μg/ml. No relationship was found between the severity of injury and growth hormone levels (C6). Other workers have not found such marked increases in growth hormone levels after surgery and conclude that changes in plasma growth hormone concentrations have little metabolic significance in the postinjury period.

The growth hormone response to intravenous glucose is increased in the postoperative period (R4). Although growth hormone is not necessary for the metabolic response to occur, it is suggested that the increased amounts in the circulation may be an attempt by the body to overcome some protein loss. However, data from patients undergoing hypophysectomy fail to show any alteration in the pattern of the catabolic response.

The effect of giving growth hormone in the postoperative period is variable. The daily nitrogen balance following herniorrhaphy was unaffected by injections of up to 10 mg/day of potent human growth hormone (J3). Others have shown a nitrogen-retaining effect of giving growth hormone for a few days after operation (C13).

Experiments in rats show that exogenous growth hormone prevents the loss of body nitrogen but does not increase the rate of healing of skin wounds (C14, C15). The mechanism of growth hormone stimulation is uncertain. Infusions of ACTH sufficient to impair glucose tolerance and

increase plasma cortisol levels have no effect on growth hormone levels (J3).

Growth hormone may play a role in the anabolic phase of the response to injury and high levels have been recorded in the serum around 10 days after trauma. An anabolic or nitrogen-retaining effect could be demonstrated at this time following the injection of human growth hormone (G4). The clinical value of artificially enhancing protein anabolism is difficult to determine. Increased amino acids in the circulation can stimulate the release of growth hormone, and this may be a stimulatory mechanism after injury.

3.3. Thyroid-Stimulating Hormone

There appears to be no significant increase in the secretion of thyroid-stimulating hormone (TSH) following injury (C6, K4). There may even be a slight fall, but the sensitivity of the methods used is probably not great enough to measure a marked decrease. The pituitary feedback mechanism for thyrotropin probably remains operative after injury in contrast to the resetting or abolition of the anterior pituitary adrenal mechanism when both hormones are present at the same time in high concentrations in the blood.

3.4. Gonadotropins

Sexual function is reduced after trauma. Men lose their libido and women experience amenorrhea until convalescence is established. Detailed reports of gonadal function at this time are scanty. Initial studies of urinary gonadotropins following injury suggest that their secretion is diminished (S6).

Female patients awaiting surgery were divided into two groups, one with normal gonadal function and the other postmenopausal. The luteinizing hormone (LH) levels did not change during surgery but fell in both groups on the first postoperative day, returning toward normal levels within 4 or 5 days (C6). Follicle-stimulating hormone levels were also unaffected during operation but likewise fell afterward. The relationship between the restoration of gonadal function and the anabolic phase of convalescence remains speculative. No constant pattern of change in LH levels was found postoperatively in another study (C2), and an early decrease was noted in only one patient.

3.5. Testosterone

It has been suggested that the protein catabolism of injury is due to the diminished activity of the anabolic hormones, testosterone and

growth hormone in the postinjury period. Measurements of serum testosterone using a double-isotope derivative method showed a consistent decrease in the plasma concentration compared to preoperative levels, which were not reached again for at least 4 days. Very high levels were recorded in some patients in the anabolic phase of convalescence (C2, C6, M3).

Studies undertaken to study the role of other endocrine activity in these changes showed that injections of norepinephrine and ACTH decreased the rate of production of testosterone and lowered the plasma level. Injections of cortisone alone had no effect on blood testosterone levels (C2).

Increased levels of growth hormone and testosterone are thus present in the blood during the anabolic phase and convalescence when nitrogen is being retained and protein synthesized. Is there an association between the high levels of anabolic hormones in blood and simultaneous protein anabolism? The administration of anabolic steroids can increase the rate of nitrogen retention during the recovery phase after surgery so that the normal anabolic response is not maximal (T7).

4. Posterior Pituitary

Antidiuretic Hormone

Normal individuals can tolerate quite large amounts of water given intravenously whereas after surgery similar infusions will produce signs of water intoxication. Oliguria commonly occurs immediately after injury or hemorrhage alone and was first described by Claude Bernard in 1859 (B4). The reduced urinary output may persist for up to 48 hours. These events are part of a primitive defense system for the conservation of water and salt which has come under endocrine control. Jones and Eaton in 1933 described unexpected postoperative edema when isotonic saline solutions were used for the rehydration of surgical patients. It was later shown that there was an obligatory retention of sodium by the body after injury and infused sodium tended to be retained (C4). Infusions of glucose 5% in large quantities were also found to cause a dilutional hyponatremia with on occasions neurological changes (C4). Sodium and water are handled differently by the kidney after operation. Water retention is more pronounced than sodium. Patients with diabetes insipidus or those who have had a previous hypophysectomy undergoing subsequent surgery have a normal retention of sodium but show no retention of water, indicating the action of multiple factors. Urine secreted after surgery is hyperosmolar with respect to plasma (E5). These clinical observations on the impairment of the handling of sodium

and water by the postoperative patient preceded the identification of any hormonal control.

Bioassay methods led to the identification of an antidiuretic hormone (ADH) in the urine of postoperative patients (C8) which was found to persist for at least 24 hours (E5).

Accurate methods of measuring ADH activity in plasma have been developed using extraction chromatographic absorption and bioassay, and values as low as 0.5 pg per milliliter of blood have been recorded (M11). This method was reproducible and allowed frequent measurements to be made before and after operation.

Apprehension and fear caused an increase in blood levels, but the induction of anesthesia was not a strong stimulus. ADH levels are often raised in the preoperative patient owing to fluid deprivation, and intravenous fluids will frequently cause a reduction in plasma ADH activity. Skin incision in a patient under general anesthesia constitutes a stimulus which can be abolished by the additional use of a local anesthetic in the skin (M6). Traction on the root of the mesentery of the small intestine was shown to be a distinct stimulus. Osmoreceptors are involved in the control of ADH release, which is inhibited when tonicity is low and is increased as tonicity rises (H12). However, after injury when the plasma is often hypotonic for many reasons and the urine concentrated, the promotion of further antidiuresis is paradoxical and unrelated to normal mechanisms of osmolality control. Plasma volume changes and associated deprivation of intake in the immediate post injury period take precedence over tonicity control mechanisms. Thus many stimuli which in themselves are not associated with blood volume changes can evoke an ADH response.

Blood loss is a major stimulus for ADH release, and after major surgery or injury raised levels have persisted in the blood for 4 or 5 days.

Elegant physiological studies showed that receptors in the ramifications of the carotid vessels and in the right and left atria transmit stimuli to the hypothalamic neurohypophysial system (V3).

Baroreceptors are present in the carotid sinus and aortic arch and stretch receptors are situated in the left atrium (G2, H6). Distension of the left atrium causes a fall in blood ADH levels, and in experimental animals the reduction in atrial stretch which follows the deflation of a distended balloon produces a brisk rise (S3). These experimental results offer an explanation of the dilutional situation with water retention which follows the surgical release of a tight mitral stenosis in man.

Vagal nerve section will abolish the ADH response to atrial and carotid stimuli and the ADH secretory response to trauma in the limb of an animal can be abolished by peripheral nerve section. The response to

stimulation of the abdominal viscera can be obliterated by regional anesthesia.

In the postinjury period an inverse relationship exists between solute clearance by the body and ADH levels in the plasma. It is suggested that ADH depresses solute-linked losses of urine by altering either the glomerular filtration rate or the renal plasma flow. It is sometimes necessary to attempt to overcome the ADH response in the postoperative period, and this can be done only by increasing the solute load to the body. Urea and mannitol will produce a modest diuresis, but the infusion of modest amounts of sodium (75–100 mEq) is the simplest method of producing more urine in the presence of excess ADH (C4, C8).

5. Insulin and Carbohydrate Metabolism

5.1. Insulin

Trauma is associated with alterations in carbohydrate metabolism, and the presence of glycosuria during surgery has been recognized for many years. Hyperglycemia and glycosuria occur soon after major injury and may persist for some days (E8, R3). Glycogenolysis and gluconeogenesis from protein sources are both increased (R3). The tolerance or rate of disappearance of both oral and intravenous glucose is reduced, but the handling of fructose is unaltered, during surgery. Starvation causes a fall in blood glucose in contrast to the rise which follows when starvation and injury are combined. Fasting blood glucose levels are always higher for several days than corresponding preoperative levels. Thiopentone anesthesia reduces glucose tolerance, but the effect is transient. ACTH infusion in the preoperative period reduced the rate of disappearance of intravenous glucose and reproduced the effect of a surgical operation (R4). The insulin response to glucose during and after major injury consists initially of a failure to respond to injected glucose which gives way within a short period to increased secretion associated with marked insulin resistance.

The insulin response to injury is a nonspecific response to stress and is similar after surgery, myocardial infarction or brain hemorrhage or hypothermia (A1).

A study in which a number of head injury patients were divided into groups depending on the severity of the injury showed a smaller rise in insulin levels in the first 3–5 days in the more severely injured. This fall in insulin levels as the severity of injury increases is probably related to catecholamine secretion. A decreased insulin response has also been observed following cardiogenic shock (D2) and hemorrhagic shock (B3).

The low insulin response in the early phase can be detected in portal vein as well as in peripheral blood (L2).

The initial suppression of insulin response is probably mediated through increased epinephrine and sympathetic activity in view of the observation that an infusion of epinephrine can suppress insulin release following a glucose infusion in normal subjects (P2). This effect can be blocked by phentolamine.

The normal insulin response to tolbutamide is suppressed in patients with cardiogenic shock; low pancreatic blood flow during shock may be a factor, but increased sympathetic nervous activity is a more likely explanation (T2).

The increased plasma insulin levels observed after glucose administration in the later postoperative period indicate that the glucose intolerance is due to insulin antagonists or resistance to insulin activity at a cellular level. The increase in plasma insulin levels are less in the elderly and in some patients with malignant disease although glucose intolerance persists (A1). Several insulin antagonists are present in the blood in high concentration after injury. Higher growth hormone levels were found during glucose infusion in the postoperative period (R4), but the rise occurred toward the end of a 60-minute glucose tolerance test so that it seemed unlikely to be responsible for the high levels recorded before and up to 40 minutes after glucose loading. ACTH infusions, although altering glucose tolerance, had no effect on insulin or growth hormone levels so that postoperative insulin levels are not related to adrenocortical activity (R4). High levels of free fatty acids are present in the plasma after injury and can exert an insulin antagonistic effect.

The insulin resistance and hyperglycemia of severe burns has been observed to persist for 1–2 weeks and has been described as pseudodiabetes. Apart from insulin antagonism of endocrine origin some cell membrane defect may be present and increased levels of the antagonist synalbumin has been found in a significant number of patients recovering from myocardial infarction (V1).

The importance of epinephrine in the changes in carbohydrate metabolism is shown by finding a close correlation between the hyperglycemia and epinephrine secretory rate in pigs. There are conflicting reports, however, on the effect of epinephrine on growth hormone secretion. The growth hormone response to arginine has been reported as abolished after epinephrine while a direct stimulatory effect of epinephrine on growth hormone secretion has also been reported.

Growth hormone rises quite quickly after glucose infusion, but no clear relationship exists. No firm conclusion can be reached on the possible stimulatory role of glucose on growth hormone.

The availability of an emergency supply of energy which is not dependent on insulin would have obvious survival value. It may be that amino acids which are immediately available after injury are a stimulus for growth hormone release which in turn increases the amount of available glucose for the emergency.

It has been suggested that the catabolic response to injury may be influenced as much by the initial absence of the anabolic hormone insulin as by increases in catabolic hormones (G5).

Plasma and urinary urea fall significantly during insulin administration in the postoperative period with a corresponding reduction in the extent of the negative balance of nitrogen. Insulin enhances the transport of amino acids into cells and their incorporation into protein. Insulin induces the production of triglyceride from carbohydrate and enhances the transport of potassium into cells. Whether or not the metabolic response is a reflection of the unavailability of insulin is difficult to determine, but there is good evidence that many aspects of the response can be modified by the administration of exogenous insulin and extra glucose, the effects not being related to glucose infusion alone (H7).

The importance of glucose in the response to injury is illustrated by noting that more than half of the hormones which are increased in the circulation after injury have the ability to maintain or raise the blood glucose level.

5.2. Glucagon

The hepatic glucose output is increased 5-fold by intravenous injection of glucagon. The maximal values are reached within 20 minutes (S2). This glucagon-induced glycogenolysis is accompanied by proportional increases in hepatic uptake of lactate and pyruvate. The output of pyruvate and lactate from the gut and hind limbs is increased in response to glucagon. These findings indicate a marked acceleration of the circulation of the components of the Cori cycle in response to glucagon with glycogenolysis and increased glucogenesis. Glucagon would appear to have an important role to play in providing extra glucose in the tissues after injury, but there is as yet no information on circulating blood levels of glucagon during or immediately after surgery.

6. Catecholamines

6.1. Epinephrine and Norepinephrine

Cannon in 1914 drew attention to the importance of increased sympathetic activity in controlling peripheral vasoconstriction and tachycardia following trauma. The secretion of epinephrine, norepinephrine,

and their metabolites in the urine is raised for 3–4 days after injury (C11, W1). The increased output represents a 10-fold increase in secretion.

Four catecholamines have been found in increased amounts in the urine after injury (W2), metanephrine, normetanephrine, *N*-methylmetanephrine, and 3-methoxytyramine. The *N*-methylmetanephrine probably indicates an increased production of epinephrine (C11). The increase in norepinephrine production is relatively greater than that of epinephrine, suggesting an active release of hormone from sympathetic nerve tissue as well as from the adrenal medulla.

Many attempts have been made to measure epinephrine and norepinephrine levels in blood after injury but no consistent changes have been detected (M9).

Some individual components of the injury experience have been studied separately in animal experiments. Pain, fear, hemorrhage, and tissue damage can all stimulate epinephrine production (W1). Inhalational anesthesia stimulates the adrenal medulla but in deep anesthesia with intravenous barbiturates adrenomedullary secretion may be greatly diminished.

The mechanism whereby the adrenal medulla is stimulated in injury has been investigated (H10). Afferent impulses arising in the carotid sinus pass to the medulla oblongata where efferent impulses are released which reach the adrenal medulla by the sympathetic nervous outflow.

Hypovolemia is a powerful stimulus for adrenomedullary secretions, and the restoration of the blood volume will reduce significantly any previously induced secretion of catecholamines (W2). Experiments in animals have shown that the response to hypovolemia does not occur if the adrenal gland has been denervated (H2). The afferent arc of this reflex pathway has not been identified.

6.2. Cortisol and Catecholamine Synergism

The functional significance of the close proximity of the sources of cortisol and catecholamines is of interest. Anatomical studies show a rich vascular network connecting the adrenal cortex and medulla in man, thus enabling high concentrations of cortisol to pass into the medulla and participate in the conversion of norepinephrine to epinephrine. Most evidence indicates that the main blood flow is from the cortex to the medulla. Steroid administration in animals produces an increased secretion of catecholamines from the adrenal medulla (H3) and stimuli which release cortisol will also release catecholamines. It may be that the blood supply of the medulla is protected during severe hypovolemia by shunting of blood from the cortex. There is good evidence of synergism be-

tween cortisol and the catecholamines and the presence of catecholamines appears to sensitize tissues to the action of cortisol. The mechanism of this synergism may be related to the electrolyte composition of cells in the walls of blood vessels as the pressor response to cortisol in patients with Addison's disease is related to sodium concentration in the plasma. It is suggested that the pressor effect of epinephrine in hypovolemia is augmented by the administration of cortisol, but direct evidence is required to substantiate this. Epinephrine releases ACTH from the pituitary in man and animals (R1).

6.3. Metabolic Implications

The metabolic effects of epinephrine are important during injury. Epinephrine can activate purine metabolism and may contribute to the increased excretion of nitrogen after injury (G9). Epinephrine and norepinephrine promote chemical thermogenesis after injury and thus will contribute to the increased metabolic expenditure of the injury period (S7). Epinephrine will also cause an acute lowering of the plasma albumin with a rise in the α-globulin fraction probably due to the effect on ACTH secretion.

The main role of epinephrine and norepinephrine after injury is probably a metabolic one. Epinephrine increases the blood flow through the liver and also the output of glucose with an associated marked increase in the uptake of lactate pyruvate and citrate from the blood. These changes can be detected within a few minutes of giving epinephrine in animals with a return to control levels within 15 minutes (H5).

The importance of the liver is shown by the failure of hyperglycemia to occur when the liver is depleted of glycogen by fasting. Either total adrenalectomy or removal of the adrenal medulla alone prevents postinjury hyperglycemia and may cause hypoglycemia.

All the metabolic actions of epinephrine appear to be directed at maintaining an adequate supply of glucose in the circulation.

7. Kidney Hormones

7.1. Renin Angiotensin

The renin angiotensin response to trauma has been investigated in some detail in reference to its controlling action on aldosterone (C9). Experimental studies in animals suggested that nerve impulses act directly on the juxtaglomerular apparatus to release renin. An intramuscular injection of sterile saline in unanesthetized rats produced an increase in plasma renin (B9). After extensive burns in man very high levels of renin were found. A detailed study on the effects of anesthesia,

surgery, blood loss and its replacement has been carried out using the method of Boucher *et al.* (B6) to estimate plasma renin. Plasma renin levels rose during surgery and fell when blood which had been lost was retransfused (B7). It may be that the role of renin is to adjust the blood volume to the capacity of the blood vascular system. Angiotensin may act quickly by vasoconstriction and slowly by stimulating the release of aldosterone to cause renal sodium retention and slow adjustment of the blood volume. The stimuli which release renin and angiotensin are similar to those which release catecholamines. The relative role of each in producing peripheral vasoconstriction is difficult to determine.

Spinal anesthesia was found to inhibit the renin angiotensin response to laparotomy in man. It would appear that renin angiotensin release is mediated by a spinal reflex arc which may be facilitated by impulses from higher centers (B8).

7.2. Erythropoietin

This hormone is thought to originate in the kidney and plays a part in increasing the production of red blood cells in the bone marrow. Increased levels of erythropoietin have been found in the plasma following trauma associated with hemorrhage. The feedback control of this defense mechanism following injury has not been investigated fully.

8. Thyroid

There has been a good deal of uncertainty as to whether or not thyroid function is altered significantly after trauma. Some aspects of the metabolic response suggest the participation of the thyroid (G7). Resting metabolic expenditure after operations of moderate severity is increased by 10% but total energy expenditure is unaltered due to a reduction in activity (T6). After severe injury metabolic expenditure and oxygen consumption are increased by more than 40% (K3). The increased breakdown of protein and oxidation of fat could also be related to an excess of thyroid hormone in the circulation. In addition, thyroxine stimulates protein synthesis and increases the incorporation of amino acids into protein (M7). Repair and protein anabolism commence immediately after injury and the thyroid hormones may be involved (G10). It is difficult to determine which changes in thyroid function are primary and due to trauma alone and which are secondary, reflecting among other things increased secretion of adrenocorticosteroids (O2). Day-to-day variations in commonly used measurements of thyroid function have also made postoperative assessment difficult. Most workers consider that the thyroid probably plays little part in the metabolic changes during injury (C16, G8).

Total serum thyroxine levels as measured by the total protein bound levels after injury have been reported as raised (F4), unchanged, or lowered (S1); but it is now agreed that there is no significant increase in total hormone levels in blood in the postoperative period.

The capacity of the thyroid to take up iodine is reduced immediately after injury (J4), and this reduced uptake persists for 2 or 3 days. The reduced uptake does not appear to be related in increased levels of ACTH, cortisol, or epinephrine, but to be a specific effect of injury (G6). Anesthesia alone produces no change. Reduced thyroid and renal clearance of ^{131}I has been found after hypovolemic shock in animals (O1). The actual iodine uptake by the thyroid falls from a mean value of 1.73 μg/hr to a mean of 0.83 μg/hr by the second day after operation. The fall rather than the rise in iodine uptake by the thyroid suggests that increased TSH activity is not increased in the postoperative period. The radioimmunoassay of TSH levels in plasma before and after surgery shows no increase and in one instance a slight decrease has been reported.

The rate of disappearance of radiothyroxine from the plasma has been reported as raised after operation. Oppenheimer and Bernstein (O1), on the other hand, found a decrease in the fractional removal of ^{131}I T_4 from plasma in the absence of a diminished degradative clearance. It has been suggested that ^{131}I T_4 may be redistributed either in the extravascular spaces or even in the gut. A significant increase in radioiodinated thyroxine in the urine has been found after injury and it is suggested that there is an increase in the peripheral degradation of thyroxine after injury (B5).

It would appear that there is a sudden increase in thyroid activity in terms of available or free hormone and an alteration in thyroxine-binding protein which starts probably during surgery and anesthesia and is associated with an increased peripheral utilization of thyroid hormone. Although changes in protein-bound iodine (PBI) and TSH concentrations are not necessarily related to secretion rates, the exact extent of any increase in secretion of thyroid hormone secretion remains uncertain.

The concentration of free rather than bound thyroxine is considered to be the most accurate assessment of thyroid activity as this is the fraction which can penetrate cell membranes and exert a metabolic effect. Free thyroxine exists in equilibrium with thyroxine bound to globulin, albumin, and prealbumin. Any changes in the concentration of thyroid binding proteins leading to an increase in free hormone. Thyroid binding prealbumin is reduced after all kinds of stress and the reduction is significant within 24 hours.

The binding capacity of thyroid-binding proteins is related directly to the concentration of the proteins in plasma. Thyroid-binding pre-

albumin (TBPA) levels fall after injury and are low when there is an increase in free thyroxine concentration (K4). The fall in TBPA after operation is due to an acute reduction of synthesis of the protein which has a very short half-life (B5, O2, S1). The increase in triiodothyronine which is recorded might be related to the fall in TBPA releasing free thyroxine which is preferentially bound to thyroid-binding globulin thus releasing triiodothyronine. It is thus possible that thyroid-binding prealbumin holds and controls the release of free T_4 for metabolic purposes in the tissues. However, there are objections to this hypothesis. Studies of radiothyroxine turnover in the postoperative period indicate that only about 15% of endogenous T_4 is bound by TBPA, and although TBPA does determine the release of free T_4 it is far less important than TBG and changes in the binding capacity of TBPA cannot account for the levels of free T_4 found in ill patients. While no change in TBG binding capacity was recorded after elective abdominal surgery (K4), others (H4) have found depressed TBG values in seriously ill patients and demonstrated an inverse relationship between TBG capacity and the free thyroxine fraction in plasma.

The change in the percentage of free thyroxine in plasma after injury is related to the severity of injury. The rise after major surgery is both more rapid and prolonged than after minor procedures. The liver plays an important role in maintaining the equilibrium of both plasma protein and the extrathyroidal organic iodine pool, one third of which is present in the liver. T_4 passes rapidly from the liver to plasma (S1). Hepatic binding of thyroxine appears to be an intracellular process rather than by the thyroxine-binding proteins in the liver. The liver will thus rapidly mop up an infusion of exogenous thyroxine. It could be argued therefore that the persistent increases in free thyroxine in the plasma after injury must be purposeful otherwise the liver would have produced a very rapid equilibrium.

Total T_3 levels in the circulation have yet to be measured after injury, but it is unlikely that there is an overall decrease in view of the finding of an increased concentration in the plasma which persists for some days after surgery (K4).

Free thyroxine levels are affected by plasma free fatty acids which are increased in the postoperative period. Normal variations and induced increases in free fatty acids produced no change in free thyroxine levels (K4).

Anesthesia alone and the stimulation of the hypothalamopituitary adrenal axis by insulin hypoglycemia or a pyrogen response failed to alter the concentration of free thyroxine.

Measurements of thyroxine secretion rate indicates a rise of 110 μg/day

in the preoperative period to 137 μg/day postoperatively. This amounts to a 25% increase which lasts for 3–4 days (H1).

The clearance of exogenously labeled L-thyroxine T_4 and L-triiodothyronine T_3 from peripheral parts is accelerated during the stress of acute infection in monkeys. The increase occurred within 8 hours and could thus not be related to any changes in binding sites. In spite of the accelerated clearance of exogenous hormone, endogenous labeled T_4 remained unchanged in sera. These findings suggest that the transport of exogenous unbound hormone into the cells is accelerated in stress (W6).

The cause and effect relationships of metabolic expenditure and thyroid activity in the post injury period await further elucidation.

9. Activation of the Endocrine Response

The stimulation of both the endocrine and metabolic response has been the subject of much study. There is little doubt that nervous transmission both from injured peripheral parts and the cerebral cortex are important initiators. Apprehension and fear can cause the increased secretion of not only catecholamines, but also cortisol and aldosterone.

Experiments in dogs showed that emotional stimulation could evoke an adrenocortical response. Impulses from the higher centers reaching the pituitary by way of the hypothalamus (E1, G1).

9.1. Peripheral Nerve Stimulation

Studies involving the use of the isolated limb (E3) have shown how minor stimuli can activate the hypophyseal system by way of peripheral nerves. Section of peripheral nerves or their tracts in the spinal cord reduces greatly adrenal secretion in response to injurious stimuli in isolated limbs. The model used in these studies was the dog with an indwelling adrenal vein catheter in which it was possible to obtain relatively basal conditions against which to measure the effect of various stimuli.

When a limb was isolated from the body apart from an artery, vein, and nerve, it was found that transection of the nerve abolished the adrenal response even though the blood supply was intact. These studies do not suggest that a wound hormone is released at the site of injury, although it is possible that histaminelike substances released in damaged tissues stimulate the peripheral nerve endings in the injured part.

The role of the peripheral nerve impulses is also shown in studies of the effect of injury in paralyzed lower limbs of paraplegic subjects (E4). Injury below the level of cord damage does not cause any increase in the levels of ACTH or cortisol in plasma (O2). Studies with spinal anesthesia do not give similar results (J2, M5). The adrenocortical response under

spinal anesthesia to surgery of moderate severity is reduced but not abolished completely (Z2).

Systemic hyperthermia alone is a powerful stimulus to ACTH release. Hypothermia has the opposite effect. Another significant stimulus is *Escherichia coli* endotoxin (E2). Patients, however, may have an adrenocortical response as a result of fever alone (R2).

9.2. Drugs and Hormones

Drugs, such as morphine, reserpine, and chlorpromazine, which are used for premedication can also cause an increased secretion of ACTH (E1, V2). The stimulatory effects of these drugs can last for many hours.

The barbiturates have little effect on pituitary adrenal activity. Ether, however, is a potent adrenocortical stimulus. This effect of ether can be overcome by giving large amounts of barbiturates.

It has been suggested that the postpituitary hormones may play an important role in antipituitary stimulation. Low doses of vasopressin had no effect on the adrenal venous concentration of cortisol when injected into the internal carotid artery but had an effect when placed in the adrenal artery (E3).

Vasopressin has a direct effect on the adrenal cortex at pharmacological doses, but it does not cause the release of ACTH at physiological concentrations.

9.3. Humoral Activators

Substances or tissue activators, such as histamine, 5-hydroxytryptamine, and acetylcholine, which are released in damaged tissue can stimulate the adrenal when injected directly into the adrenal blood supply. Early experiments in denervated limbs in rats suggested that the ascorbic acid depletion of the rat adrenal following injury in the isolated part was due to a humoral agent (G1), and recent experiments show that 5-hydroxytryptamine has a direct stimulatory effect on aldosterone secretion (J7).

Isolation of the pituitary in dogs by section of the stalk and removal of the hind brain does not inhibit a brisk adrenocortical response to injury (W5); this indicates that a humoral mechanism must be involved in stimulating the pituitary (H10). A corticotropic stimulating or releasing hormone has been isolated from the hypothalamus which appears to be epinephrine in some animals (G1), but not in man.

Experiments in which the cerebral cortices and other portions of brain were removed indicate that the cerebral centers exert mainly a suppressive influence on the activation of the pituitary adrenal axis (H11).

9.4. Oligemia

Hypotension whether induced by drugs or blood loss is a potent stimulus for ACTH release. The pituitary stimulating effect of a ganglion blocking agent which reduces blood pressure is abolished by the simultaneous administration of a vasoconstrictor substance, but the maintenance of blood pressure during blood loss does not abolish the pituitary adrenal response. The changes in blood volume produce a very rapid response which has been shown not to be due to tissue anoxia or any alteration in cerebral blood flow. Increased sensitivity of the adrenal cortex has been reported during hypovolemia (M1), but this has not been confirmed. Animals with spinal cord transection show a marked adrenocortical response to hemorrhage. Oligemia acts via carotid and aortic arch baroreceptor mechanisms to activate afferent nerve pathways to the central nervous system (S5). Vagotomy abolishes the adrenal response to hemorrhage.

Injury is made up of a number of factors, such as fear, peripheral nerve stimulation, temperature changes, anesthetic agents, endotoxins, and blood volume changes, each of which activates the pituitary adrenal axis. The hypothalamus and associated releasing factor is a final common pathway for nerve impulses from the periphery. The initial pathways involved in provoking the endocrine response are mainly but not exclusively neural. Many of the stimuli arising during operation can be modified by the maintenance of vascular homeostasis and the gentle handling of tissues.

10. Adrenocortical Insufficiency

10.1. Adrenal Failure

Abdominal surgery is a major stimulus to ACTH release, and giving further ACTH cannot further increase the responsiveness of the adrenal cortex. Immediately after operation the adrenal is capable of a further burst. The more it is pushed, the more responsive it becomes (E7).

The importance of the pituitary adrenal response to stress is illustrated by the early reports of patients with untreated Addison's disease succumbing to tooth extraction or hemorrhoidectomy.

The presence of adrenal steroids in adequate amounts is necessary for the body to recover from severe injury, and any interference with the normal pituitary adrenal relationships or diminution of the capacity of the adrenal to respond to a stimulus are of considerable practical importance. If insufficient amounts of cortisol and aldosterone are available in the tissues, peripheral circulatory failure ensues and may prove fatal.

Apart from unrecognized Addison's disease, previous ablative adrenal surgery and virilizing hyperplasia are causes of primary adrenal failure. Hypopituitarism and previous hypophysectomy are examples of secondary adrenal failure, but secondary adrenal unresponsiveness due to prior treatment with corticosteroids is much more common and is liable to prevent patients from responding normally to even minor degrees of stress (J5).

Adrenal failure has been reported more than one year after withdrawal of steroid treatment. A preoperative test to detect patients who will fail to respond to surgery is important. The adrenal may be stimulated by injections of ACTH or synthetic analogs with measurement of changes in plasma cortisol levels (M4). This test, however, only gives information about the adrenal capacity to secrete and tells nothing of the ability of the pituitary adrenal axis to respond to stress.

Carter and James (C3) studied the individual response of a group of patients on previous steroid therapy to insulin hypoglycemia, lysine, vasopressin, and corticotropin stimulation before operation and measured their plasma cortisol levels during operation. There was a close correlation between the response to hypoglycemia and surgery, and a positive response to hypoglycemia in patients treated with steroids is a good index of the responsiveness to surgical stress and indicates whether or not steroid administration is required in the postoperative period.

Factors other than an increase in circulating cortisol maintain blood pressure after injury because patients who have had steroid treatment and who fail to show any increase in circulating cortisol levels in the plasma after operation may have no fall in blood pressure.

It was considered by some that a fall in blood pressure many days or weeks after severe injuries or major surgery followed by a series of complications was an indication of adrenocortical exhaustion. Patients under such circumstances sometimes respond to cortisol therapy with an increase in blood pressure.

It is felt, however, that true adrenocortical exhaustion is a very uncommon condition (C3) and low plasma values unresponsive to ACTH are found in only a minority of very ill people (M5). The adrenal cortex usually responds to prolonged stress by hypertrophy and measurements of cortisol after prolonged stress show that they are frequently high (C12). True adrenal exhaustion or insufficiency can be diagnosed only when the plasma cortisol levels are constantly low and do not respond to ACTH stimulation.

Patients with virilizing hyperplasia of the adrenal have a relative cortisol deficiency due to a block in normal synthesis, and this deficiency may be detected only during the demands of a surgical procedure.

11. Summary

The importance of the endocrine and metabolic response is difficult to understand in the setting of modern medical care of the injured. The metabolic response appears to be wasteful of tissue protein, to cause a potassium deficiency, to predispose to the development of edema and water intoxication, and to produce hyperglycemia with ketosis. The endocrine system has an important role in permitting and controlling these metabolic events.

All the events of the posttrauma period however fall neatly into place when considered in terms of a land animal facing the problem of survival after injury. The metabolic and endocrine response can then be seen as an important biological defense mechanism.

Injury involves temporary immobilization, and so the animal cannot reach water and salt and, for survival, mechanisms are evolved to retain water and salt, and thus indirectly blood volume and the perfusion of vital organs are guaranteed. Injured tissues will liberate potassium into the circulation at a time when urine production is reduced so that an extra mechanism for potassium excretion is required to prevent harmful levels being reached. Nervous tissue has developed to a state where it requires glucose for survival except in very special circumstances, so various methods are required to meet this top priority of the vital cells for glucose.

The important question remains: To what extent should modern therapy interfere with biological mechanisms? After injury of moderate severity the metabolic and endocrine response is a physiological mechanism of adjustment and does not require any interference.

In severe injury, however, it may be important to interrupt the hypercatabolic state. After major burns energy expenditure is increased by more than 50%. Glycogen reserves are exhausted rapidly and gluconeogenesis from protein breakdown cannot provide enough carbohydrate intermediates or cope with the excessive calorie requirements. Fat reserves are mobilized and lipolysis leads to high levels of free fatty acids and fatty infiltration of the liver. In such acute situations the provision of calories, amino acids, and insulin seems logical to reduce lipolysis, spare protein, provide calories, and restore potassium to the cells. The endocrine response plays a secondary or permissive role and rarely fails unless previous suppression has occurred due to disease, surgery, or drugs.

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INSTRUMENTATION IN CLINICAL CHEMISTRY

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1. Introduction

The clinical chemistry laboratory is expected to provide a high quality and comprehensive analytical service to clinicians. Thirty years ago this service depended basically upon human manipulative skill, supplemented by simple manually operated instruments. Consequently, the range of analyses and number of tests that could be performed in a day was limited by the physical effort required from laboratory staff. The introduction of the flame photometer and photoelectric colorimeter in 1940–1950, soon followed by the ultraviolet and visible spectrophotometer, enabled more tests to be done and the range of analyses to be extended. The availability of routine chemical analyses, and the growing awareness of their value, stimulated demands for them, and laboratories were soon fully occupied in providing large numbers of about 20 different tests. The annual work load increased exponentially, producing a "period of crisis" (W16), in which many laboratories were ill-equipped or inadequately staffed to cope with the volume and range of work demanded of them.

The introduction of the AutoAnalyzer in 1957 (S13) made it possible for large numbers of many common tests to be performed speedily and accurately, without additional labor. The crisis of expanding workloads could be solved by mechanization and was limited only by the availability of funds. The AutoAnalyzer was one of the first instruments designed specifically for the needs of clinical chemistry, and it introduced a new "era of sophistication" (W16). The Sequential Multiple Analyzer (S14) soon followed and introduced a new concept: a range of tests could be performed as quickly and cheaply as a single test. Clinicians could be provided with a "profile" of information, and health screening of populations by chemical methods became possible.

The bulk of repetitive analyses can now be made by machines, and the clinical chemist is free to explore new areas using techniques and instruments not previously applied to clinical chemistry. This exploration starts in the laboratory by investigating the feasibility of a new analysis. In many cases this is determined by the availability of suitable instrumentation. If the test is feasible, and trials show that it provides clinically useful information, it will be applied routinely, and the instrumentation may then require modification or mechanization to deal with larger numbers of specimens. Relatively few analyses, once they have become feasible, have been rejected as having no clinical application; the enthusiast will always hope that an application will be found or that refinements in the method will reveal one. There is thus a danger that the mere availability of a test, particularly if performed by an instrument, will create its own demand.

The development of instrumentation has determined much of the progress of clinical chemistry. Modern hospital laboratories are increasingly dependent on complex instruments and expensive "black boxes," the principles of which may be poorly understood by those who use them. This has resulted in some fears that instruments may dictate the future work of the clinical chemist and become his master instead of his servant. There are also signs that the availability of analytical data resulting from instrumentation is outstripping its clinical understanding, and there is little opportunity for a breathing space to critically examine its usefulness.

This review is written for the clinical chemist who wishes to understand the principles of the main classes of instruments, their relative merits and applications, and the types likely to be important in the future. Equipment used for data processing, *in vivo* analysis, cell counting and morphology is excluded. Some instruments described in standard textbooks [e.g., (S15, W18)] have been omitted either because they have not developed significantly in recent years (e.g., nephelometers, refractometers) or because they have found little application in clinical chemistry (e.g., thermal analyzers).

2. General Principles of Instrumentation

2.1. The Clinical Chemist's Requirements

Several unique features dictate the type of instrument required in clinical chemistry. Large laboratories may undertake several hundred different types of analysis, some in large numbers daily and others in small infrequent batches. In most tests, the concentration of specific elements or compounds is determined, often in micro quantities. Qualitative

tests of, for example, drugs or abnormal metabolites will usually be followed by quantitative analysis. At present there is little need for instruments directed at the establishment of molecular structure, although some features of that structure may provide a basis for a quantitative method. Proteins, pigments, and cells present in biological fluids may interfere with the analysis, and consequently a preliminary separation, purification, or concentration may be necessary before a quantitative determination can be made. Samples are usually small, and it is preferable to use the same analytical procedure for both adult and pediatric patients and, if possible, for both blood and urine specimens. Chemical analysis of tissue specimens is at present mainly limited to the determination of enzymes. Instruments which necessitate solid samples are therefore of only limited application, although when these offer unique advantages (e.g., infrared spectroscopy) it may be worth isolating the compound in a solid state for instrumental analysis.

Instruments fulfill two functions: to enable an analysis to be made which is not otherwise possible, and to enable it to be made faster, more accurately, on smaller quantities, or more cheaply than by alternative methods. Having shown that the analysis is feasible, the analyst must define his instrumental requirements, decide how fast, accurate, and cheap the test is to be, and also predict the likely demand. If large numbers of specimens are anticipated, a dedicated instrument, probably mechanized or automated, may be needed, but a more versatile instrument can be chosen for smaller numbers of several different tests. The quality of any analytical result, and the cost of obtaining it, are determined by the method, the operator's technique, and the instruments used, and it is often difficult to separate the contribution of these factors. In the following sections the more important features of instrumental performance are examined in detail.

2.1.1. *Accuracy*

This may be defined as the degree of agreement between the value found and the true value. Inaccuracy, systematic error, or bias arises from nonspecificity, interference, and faulty calibration or standardization. Errors of calibration are entirely instrumental (see Section 2.3), but the other causes of inaccuracy depend also on the nature of the specimen.

The specificity of an analysis is its ability to measure solely the specified substance (W11). The function of the instrument is to isolate and measure the required signal but no others. Some chemical methods and physical parameters are highly specific for individual substances, so that little instrumental resolution is necessary to select the required signal. The nature and intensity of the unwanted signals depend on the sample,

and with less specific methods a high instrumental resolution may be required. Alternatively, unwanted substances may be removed by preliminary treatment of the sample, but this may increase costs.

Interference arises when the intensity of the signal from the required substance is modified by another substance, although the signals of the two substances are adequately resolved. Thus, the presence of phosphate reduces the flame emission of calcium by forming thermally stable calcium phosphate. The instrumental conditions can sometimes be altered to reduce the effect, or the interfering material removed by pretreatment of the sample. Standard solutions are usually chosen to resemble samples as closely as possible in the hope that any interference will occur equally in both.

The accuracy of some analyses depends on the method or instrument used, and it is now widely accepted that these differences need to be eliminated. Instruments intended for screening should not be less accurate than those used for other purposes (L1). The greatest accuracy is required at the limits of the normal range, where the concentration is sometimes small and the measurement therefore imprecise and inaccurate.

There is at present no generally accepted criterion for the accuracy of instruments, although tolerances for the calibration of volumetric glassware and thermometers have been published. Manufacturers' claims of "accuracy within 1%" are difficult to assess without knowledge of the samples tested or method used to obtain the value. It should be possible for manufacturers to specify the accuracy of calibration of many instruments. As a general rule, it seems desirable that any inaccuracy in an instrument should not contribute significantly to the total inaccuracy of the result.

2.1.2. *Precision*

Precision may be defined as the agreement between a series of replicate measurements and is usually expressed as the standard deviation (SD) or coefficient of variation (CV). The overall precision of the analysis will be determined by the errors arising from the inherent variability of the physical methods and chemical reactions used, as well as those introduced by instruments. Analytical technique will also contribute to this variability, but one of the purposes of good instrumentation is to reduce the influence of technique and analytical skill which, when applied to repetitive manipulations, are variable and unreliable factors.

Instrumental precision is determined by the stability of the analytical signal and is made up of two components—noise, appearing as a random variation, and drift, which is a systematic change in signal level. The importance of drift will depend on the frequency of standardization and

the rate of analysis; with fast analytical rates, drift becomes less important. With many instruments, the precision depends on signal intensity and is poor at the extreme limits of the scale. It would be helpful if manufacturers could specify the optimum operating range of their instruments so that the analyst could use this for his most critical measurements. Sample interaction or carryover occurs when one sample is contaminated by a previous one during the analysis (T3). It can occur in any instrument where successive samples follow the same path, and it will result in a deterioration in precision. Methods have been described for its measurement (B17), and corrections can then be made to the analytical results.

The precision required for clinical analyses has been expressed as the "tolerable analytic variability" (Y2), and for many tests it is equivalent to a CV of less than 1%. The precision required from an instrument will therefore be less than this, depending on its contribution to the overall variability.

2.1.3. *Sensitivity*

Sensitivity is the ability to detect small changes and depends on the response of the system to change of input and the stability of the signal. The detection limit is defined as the smallest single result which, with a 95% probability, can be distinguished from zero. It is determined by the stability (noise) of the background signal (blank). The limit may be a concentration or amount of substance and defines the point at which the analysis becomes just feasible. However, since the signal is small, and only just detectable, the precision of measurement will be poor (CV ~50%). A linear calibration curve may become nonlinear with large signals, and ultimately further increases in concentration produce no increase in signal. The analytical range is defined as the range of concentration, in the original sample, over which the method is usable with acceptable accuracy and precision. If concentrations in normal subjects are to be measured precisely, the signal must be relatively large, and abnormal high concentrations may then be outside the analytical range. Consequently, it is often necessary to compromise between high precision and a wide analytical range.

Sensitivity is increased by amplification and by reducing noise; methods of manipulating the signal to achieve this are discussed in Section 2.2.

2.1.4. *Speed*

Speed is necessary not only on clinical grounds, but also to reduce the costs arising from labor and equipment. The throughput time is the time

taken for a single analysis—that is, the interval between sampling a specimen and the output of the final result. The rate of analysis is the number of analytical results, for any one component, which can be produced per hour. With automated systems, this rate is fixed and for different machines may be between 30 and 300 per hour. If the analytical work of the laboratory was evenly spread throughout the day, lower rates would be acceptable, but one of the advantages of fast systems is that they can deal with a sudden influx of specimens at peak hours. Although in emergencies it may sometimes seem justifiable to sacrifice accuracy and precision for speed, this is a dangerous principle to apply to instruments, as it is unlikely that the instrument will be reserved for emergencies only.

2.1.5. *Cost*

The true cost of an analysis is almost impossible to calculate owing to the imponderable nature of many overheads and the difficulty of apportioning these between different tests. Calculations are usually limited to the direct costs of labor, equipment, reagents, and consumables, and these permit the relative costs of different procedures to be estimated.

The annual cost of equipment includes depreciation and maintenance. In addition, an allowance should be made for the loss of interest which, if the instrument had not been purchased, would have accrued from investment of the capital. Depreciation is the difference between the capital cost and the secondhand value. In practice, the secondhand value of an instrument is usually small, not because it is worn out, but because there are newer and better instruments available for performing the same task. Few instruments cannot be replaced by faster and better ones after 5 years, although many continue to be used for long after this time despite progressively increasing maintenance costs. This "built-in obsolescence" makes rental attractive as this enables the user to change his instrument quickly without loss of capital; it may also act as an incentive to manufacturers to improve their maintenance service.

To simplify these calculations, the capital cost of the instrument may be amortized over 5 or 6 years and maintenance costs ignored. The average daily cost can then be calculated and will be the same whether the instrument is used or not. Reagent costs are simple to calculate and are usually small in relation to other costs. Examples of labor and equipment costs of 5 commercial flame photometers, used to measure plasma sodium and potassium simultaneously, were given by Broughton and Dawson (B18). With small numbers of analyses, the least expensive instrument was the cheapest to run, but despite wide differences in capital outlay and labor requirements, the cost per analysis for the 5 instruments

operated at more than 100 analyses per day was not markedly different. Labor costs are constantly increasing in most countries, so that it is more economical to purchase a more expensive instrument if this reduces the time spent by the operator on the analysis.

Few detailed investigations have been published of the relative costs of labor and instrumentation or of the costs of alternative procedures in clinical chemistry. With the continuing growth of analytical work and the high price of instruments, precise costing will become increasingly important. In order to obtain more information about this, it will be necessary to define costing methods which can be applied to different procedures, and to devise suitable methods of work study (P9). A cost benefit-analysis, such as that made in one laboratory which installed automation and data processing (K4), is a salutary exercise for laboratory managers if it can be made in quantitative terms. Agreed methods for doing this are urgently required.

2.1.6. *Instrument Evaluation*

It is surprisingly difficult to chose an instrument merely by studying manufacturers' specifications and having a "trial run" with a few alternative models. Manufacturers' literature may not describe adequately the particular function of interest to the clinical chemist, and performance data may be difficult to interpret or overoptimistic if obtained under the ideal conditions of the manufacturers' laboratory. The experience of someone who has already purchased the instrument is not always a good guide, since he may be unwilling to admit that he made a bad choice. Consequently the user may wish to evaluate alternative models. Since there are few objective criteria of performance, he can only judge one instrument in relation to another. To do this thoroughly is extremely time-consuming and during the last few years many evaluation reports, made in clinical laboratories and independent of the manufacturer, have been published.

Before any evaluation, the essential characteristics required of the instrument must be defined. These will include accuracy, precision, specificity, and speed, and methods for measuring these may need to be devised. To these objective criteria must be added a range of subjective factors, including ease of use, reliability, safety, and ease of maintenance. Finally, all these factors must be related to the price of the instrument. In Britain a schedule for testing automated equipment has been prepared (B17) which with minor modifications can be applied to many different types of instrument.

The results of most evaluation studies have revealed deficiencies in individual instruments and sometimes in groups of instruments, which

have often resulted in their modification or withdrawal from the market. Studies of 9 simple colorimeters showed many design faults, with drift of readings and nonlinear responses (B20), and it was concluded that none of the instruments was as good as it should be for use in a clinical laboratory. Comparative tests on 35 commercial automatic dispensing pipettes showed that many were fragile, difficult to use or expensive, and the manufacturer's calibration was sometimes inaccurate (B19). The precision obtained was often dependent on the technique of the operator, a factor not usually mentioned in the instructions. These and many other studies have shown that the most expensive instruments are not necessarily the best and that manufacturers' claims for the performance of their equipment were sometimes not substantiated when the instrument was tested independently. Usually the choice of an instrument is a compromise between its performance and price, and the user must decide on the level of performance suitable for his purpose, at a price he can afford. At present, information of this type is extremely difficult for the average clinical chemist to obtain. The evaluation itself and dissemination of the results take time, and frequently the instrument has been modified or sold in large numbers before the results are available.

2.2. Signal Manipulation

An instrument acts as a communication device that converts chemical or physical information into a form which is more readily observed (S15). It does this by (1) generating a signal which is as large and stable as possible, (2) transforming the signal to one of a different nature, (3) amplification, and (4) presentation of the final signal in a readable form. The main factors involved in these processes are outlined below.

2.2.1. *Noise*

Noise is defined as any unwanted disturbance in a required signal. All electrical signals are basically unstable and noise may arise either from the instrument itself or from the fundamental process generating the signal. Shot noise is due to the variations resulting from the quantum nature of energy, as in the statistical fluctuations in the flow of electrons to an anode. Flicker noise is a consequence of instabilities in the procedure used to activate the source of energy, as when variations in the nature of the cathode surface affect the rate of emission of electrons. Shot noise is completely random and has a white power spectrum—that is, a uniform energy versus frequency distribution—whereas flicker noise has strong frequency components. The signal-to-noise ratio is the ratio of the power available in the form of the required information to that

present in the accompanying noise. The effect of noise is to increase the detection limit and reduce the precision of measurement.

Many sources of noise can be overcome by careful design, such as the use of shielding to prevent pickup of random voltages, smoothing of power supplies to obviate the effect of main voltage variations, and the use of shock absorbers to reduce mechanical vibration. Other sources of noise can be avoided by simple precautions, such as avoiding defective components, poor contacts, leaky insulations, and temperature fluctuations which adversely affect the noise of amplifiers and photomultipliers (W8).

If the signal of interest is superimposed on a strong background signal, the latter will contribute a proportionate amount of noise. When measuring very small signals, any background should be made as small as possible by, for example, reducing the background count in radioactivity measurements, stray light in a spectrophotometer, or dark current in a photomultiplier. Since noise is random, its average value will be zero, so if a longer observation period is used a larger signal-to-noise ratio will be obtained in the accumulated data. Averaging the signal by damping or the use of devices such as integrating networks improves the signal-to-noise ratio (W8). Flicker noise is best overcome by frequency modulation of the signal—that is, superimposing a different but known frequency on the source of the noise, for example by inserting a chopper in a light path, and then using a frequency noise filter to select the modulating frequency and reject the unwanted noise. Small signals are easier to separate from a large background if they are modulated.

2.2.2. *Amplification*

Before signals generated by the detector can be displayed, they frequently need to be modified by amplification, rectification, demodulation, or integration. An amplifier increases the magnitude of a signal by a factor known as the gain. With dc amplifiers both the signal and noise will be magnified but, with a modulated signal, filtration can be incorporated in the amplifier to reduce the proportion of flicker noise. The use of narrow-bandpass amplifiers can greatly improve signal-to-noise ratios, but as some potentially useful information may be lost the observation period may need to be increased. Precautions must be taken to ensure that instabilities in the amplifier itself do not introduce noise.

Scale expansion is used to increase the apparent magnitude of a signal in order to make precise readings easier. This is achieved by increasing the sensitivity of the measuring system and "backing off" a major portion of the signal with a constant current or voltage of opposite polarity. The

signal and noise are equally magnified by scale expansion, and an increase in the damping or integration time may be necessary to give a stable reading.

2.2.3. *Display*

A transducer is a device for converting signals from one form or medium to another (W8). For example, a photocell transforms a light signal into a current or voltage signal which the output transducer presents as a meter reading, chart recorder trace, or digital display. The precision of measurement depends largely on the characteristics of the readout system. Errors can arise from hysteresis in a galvanometer, parallax in the reading of a meter needle, and inertia or bias in a pen recorder. Manual reading of a meter deflection is the least precise method, whereas printout is the most precise and the most expensive. In null point methods, the operator is required to back off the signal until a meter returns to its zero position. Since the result found will be independent of the response of the measuring system, this method is more precise. In general, digital signals, such as those produced in radioactivity measurements, are easier to manipulate with modern data-handling equipment. Simple electronic aids have been developed for converting peak heights of chart records into a printed display in concentration units (D6).

Until recently, the signal displayed by most instruments has required mathematical treatment to obtain the required result. Now, however, calibration factors are frequently built into the instrument so that the digital voltmeter or printout gives the required result. There is some danger that this may induce a false sense of security in the analyst and a reduced awareness of the limitations of the basic analytical process, which can be avoided only by continued quality control.

2.3. Calibration and Standardization

Calibration may be defined as the relationship between the true value of a measured quantity and the corresponding scale reading. Thermometers, pressure gauges, and wavelength scales are calibrated by the manufacturer in precisely defined units (e.g., degrees Celsius, pounds per square inch, and nanometers, respectively) and should register true values. However, most instruments produce an analog signal (e.g., a galvanometer deflection) which must be compared with a standard in order to obtain a result in the required units. A galvanometer scale of a spectrophotometer may be calibrated by the manufacturer in absorbance units, but the analyst must use a standard to relate the absorbance to concentration.

At least two points on the scale must be defined—a zero value and a full-scale value. In quantitative analysis a linear relationship between signal strength and concentration is desirable, as this simplifies calibration and calculation. Inaccuracy will result if calibration and standard curves are incorrectly assumed to be linear. Nonlinearity may be inherent or due to an instrumental fault but can always be checked with a range of standards. When the relationship between signal strength and concentration is reproducible but nonlinear, several different methods may be used to linearize the calibration curves. The absorbance scales of some spectrophotometers are divided logarithmically. Alternatively the signal may be modified by, for example, using a logarithmic shaped wedge in the light path or a photocell with a logarithmic response instead of a linear one. With more complex relationships, linearizing circuits, such as function-generating networks, or a computer can be used to produce an output which is directly proportional to concentration. The choice between these methods depends on the constancy and complexity of the curve and the price the user is prepared to pay for linearization.

In most calibration procedures, samples and standards are analyzed at different times, and errors arise if the instrumental sensitivity varies. An internal standard may be used to correct for this effect. This is a substance, not normally present in the sample, and clearly distinguishable from the compound sought, which is added in constant amount to both sample and standards. The ratio between the signals produced by the internal standard and the required substance is recorded. It is then assumed that any change in sensitivity will influence both signals equally, so that their ratio remains constant. Isopropanol is frequently used as an internal standard in the determination of ethanol by GLC; both compounds are measured with the same detector and their peak heights should therefore be equally affected by changes in sensitivity. The lithium internal standard used in flame photometry usually requires a separate detector. The lithium will compensate for changes in sensitivity due to variations within the flame, but not for changes in the sensitivity of the two photocells when these differ. Lithium may also interfere with the analysis by suppressing the ionization of the sodium or potassium being determined. One report (B18) noted little difference between the performance of flame photometers employing a lithium internal standard and those which did not. The best internal standard is one containing an isotope which will behave in exactly the same way as the substance being analyzed but which can be measured independently (W11). In the "standard addition" method, standard solutions of the substance being analyzed are added to the sample and the increase in signal plotted against the amount added. The sample concentration can then be ob-

tained by extrapolation, but the method is only of use when the shape of the calibration curve is known and preferably linear.

Errors in calibration and standardization result in poor accuracy, and are considered in Section 2.5.

2.4. Mechanization and Automation

Mechanization may be defined as the use of instruments or other devices to reduce or replace human effort. Automation fulfills the same task but in addition replaces the human faculties of observation and decision. The operator initiates the process which is thereafter self-controlling and self-correcting; signals are fed back into the system to control it so that, once started, no further human intervention is required. True automation has so far been rarely achieved in analytical instruments, most of which are best described as mechanized.

Most analytical methods require several separate instruments or modules, each with a specific function. Frequently these are mechanized to reduce the amount of physical effort required—for example, samplers, turntables, automatic pipettes, and colorimeters linked to recorders or small printers. The application of simple instruments and homemade work-aids of this type, coupled with simple ergonomic principles, has proved rewarding with manual methods and leads to improved precision and faster analysis, with reduced operator fatigue and boredom (M14). There is still considerable scope for this type of work simplification to reduce the number of steps in any manual operation. When a number of instruments or modules are linked together, they form a system, which will analyze a series of specimens. Systems may either operate continuously, analyzing a batch of specimens without interruption, or discontinuously, where the operator is required to initiate each stage of the analysis and transfer specimens from one stage to the next. A group of modules or components involved in one analytical method is termed a channel (B17). A single-channel system will, without modification, analyze each specimen for one constituent. In multichannel systems two or more different analyses are made concurrently on the same specimen. This is achieved by splitting the specimen, or signals derived from it, at some stage in the analysis. In clinical chemistry the analytical process starts with the receipt of a blood specimen and finishes with the production of a report of the analytical results. Some systems incorporate printers, or are linked to a computer to produce a printed report, but none are completely self-controlling, although some include alarm devices and accessories for self-standardization.

The problems of enzyme analysis provide a good example of the development of an automated system. There are three stages in the auto-

mation of enzyme assay by kinetic methods (S3): (1) preparation of the reaction mixture, (2) recording the effects of enzyme action, and (3) conversion of the signal into a numerical value expressing the activity of the enzyme. The first two of these stages can be accomplished separately using mechanized instruments. Trayser and Seligson (T10) described a method for combining the second and third stages, using a double-beam spectrophotometer to measure the reaction rate, which is proportional to the enzyme activity. Complete automation will be achieved when these three stages are linked together and the output passed to a computer which will analyze the data, feed back information to control the analytical instruments, and finally print the report.

Most commercial automated systems have been developed for the limited range of analyses which are performed in large numbers. Nevertheless there is considerable potential for mechanizing part or all of many types of test, particularly complex analyses made on small numbers of specimens. No attempt will be made here to discuss the details of current commercial systems, as these are adequately described elsewhere and most are still developing rapidly (B15, G2, G10, K9, L1, M13, M14, M15, N6, N7, W15). The majority can be classified into one of two types: (1) continuous flow systems, where solutions derived from succeeding samples all flow along the same path throughout the entire analytical process (B17), (2) discrete systems, where solutions derived from different samples are contained, during part or all of the analytical process, in separate vessels (B17). Both systems may suffer from sample interaction and instrumental drift and require some degree of supervision. Sample blanks are not usually required in continuous flow systems, since proteins are removed by dialysis, and this enables some tests to be made on whole blood specimens. Most discrete systems are limited to analytical methods which do not require removal of protein. Manual methods are easier to apply to discrete systems, but the large number of moving parts requires a high degree of mechanical reliability. Discrete systems can be operated at speeds up to 300 specimens per hour, whereas continuous-flow systems are normally limited to not more than 100 per hour, although the speed can be increased by applying a curve regeneration device to the recorder output (W1), or using a computer to correct for sample interaction (T3). The recorder used in continuous flow systems provides a useful method of monitoring analytical performances. The complexity of the larger multichannel discrete analyzers necessitates an on-line computer to monitor and control the analysis.

Two major problems with both types of system are standardization and sample identification. With multichannel instruments a serum standard or reference is almost obligatory. This must be standardized

by independent reference methods using pure aqueous standards. Units of enzyme activity are defined in terms of reference methods, but some automated systems use different methods, sometimes with suboptimal conditions. Most current discrete and continuous flow analyzers determine enzyme activity by single-point measurement. Some clinical chemists believe that it would be better to perform all enzyme determinations on independent automated systems designed specifically for enzyme analysis and using kinetic methods. Although many analytical systems include devices for sample identification, none yet appear to be sufficiently cheap or foolproof. Gambino (G2) has suggested that a lock and key approach is required, so that it is impossible for a specimen or result to be ascribed to the wrong patient. One of the objectives of new fast analyzers is to make the analysis sufficiently fast and cheap that all tests can be performed in replicate, thus minimizing the chance of mismatching specimens and results (A7).

Both discrete and continuous flow systems operate sequentially, so that results on standards and controls are output at different times from those on patients' specimens. True automation requires a method of monitoring a reaction throughout its entire course to ensure that it is proceeding satisfactorily and that reagents, volumes, temperatures, and times are correct. Analytical results on control specimens need to be fed back into the system in time for a computer to detect a fault, take corrective action and ultimately control the analysis. This can be achieved only if data are produced at intervals measured in milliseconds and fed directly into a computer, and this necessitates analyses in parallel (i.e., simultaneously) instead of sequentially (A7, A8, H7). The GEMSAEC system is the only one yet developed to use this principle (A9). It is based on the use of centrifugal force to move and mix samples and reagents concurrently. The rotor is positioned so that its cuvettes spin between a light source and a photomultiplier, and the absorbances are displayed on an oscilloscope. The rate of analysis is proportional to the number of cuvettes in the rotor and machines are available with up to 42-place rotors, enabling analytical rates of up to 160 samples per hour to be achieved (B23). The instrument requires 2.5–50 μl of sample and is economical of reagents, so its running costs are likely to be small. Although it normally functions as a single-channel instrument, it has been used for the simultaneous assay of three different enzymes in serum samples (T6). The instrument is being applied to a variety of analyses, but seems particularly suited to the determination of enzymes by measurement of reaction rates.

The performance requirements of both mechanical aids and automated systems are similar to those of all analytical instruments (see Section

2.1)—that is, accuracy, precision, sensitivity, and speed, all of which must be balanced against cost. Although the performance of individual systems is well documented, there have been surprisingly few detailed comparisons of the costs of the same analyses made on machines with similar and satisfactory performance. The degree of mechanization and automation which is justified depends primarily on the work load. With work simplification, large numbers of tests will require more labor, and possibly extra laboratory space, thereby increasing running costs. Automated instruments are more compact but large capital investment is economic only if the equipment is efficiently used. In selecting a suitable instrument, one of the most difficult decisions is the degree of flexibility required. Mechanical aids and most single-channel machines are flexible, but require more labor. Inflexible or dedicated instruments are justified for tests which are certain to be required in large numbers and urgent specimens can then be handled without waiting for an instrument to become available. Most multichannel instruments are basically inflexible and the user must decide which tests are to be included before buying. In many, the operator cannot modify the chemistry if he wishes. Some can be operated in a discretionary mode, where the operator selects the tests required for each specimen. This method appears to be expensive, since a full range of tests would need to be provided and there would be a natural tendency to ask for all of them. In choosing between a multichannel instrument and a series of single channel machines, the total laboratory costs (per *specimen,* not per *test*) will probably be the main factor; the larger capital cost of multichannel equipment must be balanced against the cost of the additional labor and inconvenience of operating single channel machines. Multichannel instruments are more economical of specimen and produce more information, although it is at present impossible to cost the value of this to the physician.

The future development of automation depends largely on the integration of analytical systems with computers, which are outside the scope of this review. Seligson (B15, W10) has suggested that data acquisition and processing systems will determine which instruments will survive in the modern laboratory. The computer can be regarded as an extension of the analytical instrument and machines which cannot be linked with the computer will become obsolete. The development of new fast automated systems has created new bottlenecks and Gambino (G2) has suggested that more attention should be directed to automatic or self-sampling of blood specimens, their faster transport to the laboratory, improved methods of plasma separation, followed by automated presentation and evaluation of the report.

2.5. Quality Control

The inherent accuracy and precision of instruments have a profound influence on the quality of the results they produce. In addition, instrument malfunction is now recognized as a potential source of error, and one survey ascribed 18% of the errors detected as due to equipment (W17). Such errors are usually detected by quality control of the complete analysis, but identification of the cause is more difficult and is often unsuccessful because of the lack of suitable methods of quality control of instruments. If these were applied as part of regular maintenance, errors would undoubtedly be identified more rapidly and before they significantly affected the analysis.

Instrumental errors have two primary causes: (1) faulty calibration, either by the manufacturer or developing during use, and (2) malfunction, often abetted by poor technique. The methods used to detect both types of error depend on the instrument, but some examples may illustrate the need for additional methods and encourage their wider routine application. The accuracy of thermometers, clocks, and volumetric glassware is rarely checked in the laboratory, and the manufacturers' calibration is assumed to be correct. Reassurance is easily given by, for example, comparing the readings of two thermometers. The calibration of pipettes may be incorrect (B19). The wavelength of maximum transmission of identically marked interference filters can vary by as much as ±6 nm; in one filter an error of 9·nm from the nominal value resulted in magnesium interfering with the determination of calcium (H1). Errors such as these are clearly the manufacturers' responsibility and until accurate calibration can be guaranteed or independently certified, the analyst will occasionally have cause to regret that he took this for granted.

Different operators, using the same instrument, may obtain different results due to variations in technique which, for example, markedly affects the precision of some automatic pipettes (B19). Manufacturers' instructions may give little or no information on how to obtain the best results. The optimal absorbance required to obtain maximum precision varies for different types of spectrophotometer from 0.43–0.88 (H26); the user may not know this if it is not stated in the instructions. Inadequate maintenance is undoubtedly a major source of error, and includes such simple faults as greasy spectrophotometer cuvettes and pipettes and dirty tubing in continuous-flow systems, resulting in excessive sample interaction. Errors of spectrophotometers, arising from poor technique and faults in wavelength accuracy, photometric linearity, and photometric accuracy, are discussed in Section 4.5.

The increasing dependence of clinical laboratories on sophisticated instruments is creating new problems in quality control for both the manufacturer and user. A good example is the difficulty of verifying the accuracy of at least one commercial reaction rate analyzer used for enzyme analysis, where standards are not available and the result is calculated from the rate of change of absorbance at 340 nm. Since the instrument does not record absorbance, but only its change, photometric accuracy cannot easily be checked. No means is provided for verifying the cuvette temperature, and the manufacturer's setting for this, together with the accuracy of the filter wavelength and the recorder chart speed, must be assumed. In situations like this, it seems essential that either the user should be able to check the accuracy of settings or the manufacturer must guarantee them.

3. Atomic Spectroscopy

Analytical applications have been found for all parts of the electromagnetic spectrum ranging from microwaves through visible radiation to gamma (γ) rays (Table 1). The emission and absorption of electromagnetic radiation are specific to atomic and molecular processes and provide the basis for sensitive and rapid methods of analysis. There are two general analytical approaches. In one, the sample is the source of the radiation; in the other, there is an external source and the absorption or scattering of radiation by the sample is measured. Emission from the sample may be spontaneous, as in radioactive decay, or stimulated by thermal or other means, as in flame photometry and fluorimetry. Both approaches can be used to provide qualitative and quantitative information about the atoms present in, or the molecular structure of, the sample.

The wavelength or quantum energy of the specific radiation giving rise to the analytical signal must be known. This information may be implicit in the nature of the radiation source employed (i.e., it is mono-energetic), but more commonly spectral analysis is required to separate the analytical signal from other signals generated by the source or sample. Spectral analysis is effected either by selecting the required radiation and measuring its intensity with a quantum energy insensitive detector, or by converting the radiation into a quantum energy dependent electrical signal.

A nondispersive system of monochromation filters either the radiation or the electrical signal generated in the detector. In a dispersive monochromator, radiation is separated according to wavelength. The resolution of a monochromator is defined as $\lambda/\delta\lambda$, where $\delta\lambda$ is the minimum distance between the centers of two spectral lines which can just be dis-

TABLE 1
THE ELECTROMAGNETIC SPECTRUM

Wavelength (m)	Frequency (Hz)	Wavenumber (cm^{-1})	Energy E (eV)	Radiation	Spectroscopy
1.2×10^{-13}	2.4×10^{21}	8.1×10^{10}	10^{7}		
				γ-Ray	γ-Ray emission
1.2×10^{-11}	2.4×10^{19}	8.1×10^{8}	10^{5}		
				X-Ray	X-Ray emission, absorption
1.2×10^{-9}	2.4×10^{17}	8.1×10^{6}	10^{3}		
1.2×10^{-7}	2.4×10^{15}	8.1×10^{4}	10^{1}	Ultraviolet	Vacuum UV absorption
				Visible	UV and visible absorption, emission, and fluorescence
1.2×10^{-5}	2.4×10^{13}	8.1×10^{2}	10^{-1}	Infrared	IR absorption, Raman
1.2×10^{-3}	2.4×10^{11}	8.1×10^{0}	10^{-3}	Microwave	ESR; Microwave absorption
1.2×10^{-1}	2.4×10^{9}	8.1×10^{-2}	10^{-5}		
1.2×10^{1}	2.4×10^{7}	8.1×10^{-4}	10^{-7}	Radio	NMR

tinguished as separate entities and λ is the mean wavelength of the two lines.

The intensity of the selected radiation is measured by devices in which the radiation either interacts with the materials in the detector (e.g., electron release, thermal heating) or generates induced currents due to the electromagnetic field of the radiation.

Samples are presented for analysis as solids, liquids, or gases, at high or low temperatures, or they may be transformed from one phase to another during the analysis. Some samples require extensive pretreatment to concentrate the analyte or to remove interfering materials. The time required for pretreatment is usually much greater than that needed for the analytical measurement, and hence, if an expensive instrument offers greater specificity than its rivals, the reduction in pretreatment costs may more than compensate for the higher initial cost.

Spectrochemical analysis provides a method for identifying and measuring elements by their emission or absorption of electromagnetic

radiation. The specificity of these methods arises from the uniqueness of the wavelength and the narrowness of the spectral line characterizing the element. As the processes which give rise to energy absorption and emission in the 180–900 nm range take place in the free atom, the sample must be converted to an atomic vapor.

The energy (E) of the photon emitted or absorbed when an electron moves from one energy level (E_0) to another (E_1) is given by the equation

$$E = E_1 - E_0 = h\nu = \frac{hc}{\lambda} \tag{1}$$

where h is Planck's constant, ν the frequency of the radiation and c the velocity of light. The wavelength of the radiation (λ) associated with any particular electronic transition is thereby exactly defined. The width of the line lies between 0.001–0.01 nm (D10) and depends on the uncertainty of the energy level, splitting of the energy level and broadening due to Doppler, pressure, and self-reversal effects. The intensity of a line is a function of the number of atoms in the excited state, the time for which they remain excited and the probability of the electrons returning to the ground state by the transition corresponding to the particular spectral line. The more intense lines arise from the commoner transitions and atoms with a single valency electron give the highest intensities. A few lines in the emission spectrum are reversal lines; that is, if the atom is exposed to radiation of that wavelength, a photon can be absorbed and an electron raised to the appropriate energy level. This is the fundamental process in atomic absorption and fluorescence analysis. The radiation emitted in fluorescence analysis may be of a different wavelength from that absorbed (W24).

The number of excited atoms N_1 of energy E_1 is given by the expression:

$$N_1 = N_0 \frac{P_1}{P_0} \exp \frac{-(E_1 - E_0)}{(kT)} \tag{2}$$

where N_0 represents the number of atoms in the ground state of energy E_0; P_1 and P_0 are the statistical weights of the excited and ground states, respectively, and k is Boltzmann's constant. The effective excitation temperature (T) depends on the mode of exciting the particular energy level and, for a flame or plasma, may differ from one element to another (A1). It is apparent from Eq. (2) that the number of atoms in the excited state is proportional to the total number of free atoms and is also dependent upon temperature. A rise in temperature, therefore, gives increased sensitivity in emission analysis unless it causes appreciable ionization. With elements of low excitation energy, such as sodium and potassium ($E \approx$

2 eV), a temperature of 2000°K excites about one in 10^5 of the total atoms present at any instant and these can be readily estimated by emission flame photometry. At the same temperature elements such as Mg and Zn with high excitation energies ($E \approx 5$ eV) have only one in 10^{14} of the total atoms present in the excited state, and they therefore have low emission sensitivities. They are, however, very suitable for estimation by atomic absorption or fluorescence, where the sensitivity depends on the number of atoms in the ground state and the oscillator strength of the transition (W2). Alternatively, such elements can be estimated satisfactorily by emission methods using the much higher excitation temperature attainable in, for example, the electric arc or radio frequency (rf) plasma (F2).

If an atom absorbs sufficient energy (5–10 eV), a valency electron may be ejected and ionization occur. The spectrum of the ionized atom is different from that of the neutral atom. If emission methods are used for an element with a high excitation potential and the sample contains other elements with low excitation and ionization potentials, considerable suppression of sensitivity may result from competition for the available energy. According to Alkemade (A3), the relative sensitivities of atomic emission and absorption processes are in the ratio of the spectral radiances, at the given wavelength, of blackbodies at the temperature of the flame and the absorption light source. This relationship has been confirmed experimentally (D7). These studies show that for an atomic vapor at 2400K and light source at 6000K, emission is equal or better in sensitivity than absorption for wavelengths greater than 350 nm. To obtain detection limits in fluorescence comparable with those of absorption, the atomic vapor must be irradiated with a least 100 times more light.

3.1. General Instrumental Considerations

Emission analysis requires simpler instrumentation than either absorption or fluorescence but with conventional flames it is limited to elements with analytical lines of wavelength greater than 300 nm. The use of higher temperature flames (>3000K) and plasmas complicates the instrument but increases the analytical capacity of the system by improving atomization and excitation. The low background emission of shielded or separated flames (H18) and nonflame cells (K10) permits very low detection limits to be obtained using fluorescence. In atomic absorption the signal is large and hence the technique can be used throughout the range 180–800 nm. Emission and fluorescence are, however, more suitable for simultaneous multielement analysis. Detection limits for a number of elements of clinical interest are given in Table 2.

TABLE 2

DETECTION LIMITS IN ATOMIC SPECTROSCOPY FOR SELECTED ELEMENTS OF BIOLOGICAL INTEREST

Element	Serum conc. (g/ml)	Method[a]	Detection limits[b]					
			Emission		Absorption		Fluorescence	
			g/ml	g	g/ml	g	g/ml	g
Ca	10^{-4}	F, N	10^{-10}		10^{-10}		10^{-8}	
		P, W		10^{-11}				
		S		10^{-8}				
		C				3×10^{-12}		
Cd	10^{-8}[c]	F, N	10^{-7}		10^{-9}		10^{-12}	
		P, N	3×10^{-6}					
		F, Sb				10^{-11}		
		C				10^{-13}		1.5×10^{-12}
Co	10^{-9}[c]	F, N	3×10^{-6}		7×10^{-9}		5×10^{-9}	
		P, N	3×10^{-9}					
		C				6×10^{-12}		
		S		5×10^{-8}				
Cr	3×10^{-8}	F, N	6×10^{-9}		5×10^{-9}		10^{-8}	
		P, N	10^{-9}					
		C				5×10^{-12}		6×10^{-12}
		S		5×10^{-9}				
Cu	1.1×10^{-6}	F, N	3×10^{-8}		3×10^{-9}		2×10^{-9}	
		S		2×10^{-8}				
		C				7×10^{-12}		10^{-12}
Fe	1.2×10^{-6}	F, N	3×10^{-8}		5×10^{-9}		10^{-7}	
		P, N	5×10^{-9}					
		C				3×10^{-12}		3×10^{-9}
		S		2×10^{-8}				
Hg	5×10^{-9}[c]	F, N	1.2×10^{-4}		3×10^{-7}		10^{-7}	
		P, R		2×10^{-9}				

		S		2×10^{-7}				
		F, Sb				2×10^{-8}		
		R				2×10^{-10}		10^{-9}
		C				10^{-10}		
K	1.7×10^{-4}	F, N	2×10^{-10}		3×10^{-9}			
		P, W	3×10^{-12}					
		C				10^{-12}		
		S		10^{-8}				
Li	2×10^{-3} [c]	F, N	3×10^{-12}		2×10^{-9}			
		C				5×10^{-12}		
Mg	2×10^{-5}	F, N	10^{-8}		3×10^{-10}		10^{-8}	
		P, N	10^{-9}					
		C				6×10^{-14}		10^{-13}
		P, W		3×10^{-11}				
		S		10^{-9}				
Mn	10^{-8}	F, N	10^{-8}		3×10^{-9}		5×10^{-8}	
		S		2×10^{-9}				
		C				5×10^{-13}		5×10^{-12}
Mo	5×10^{-9} [c]	F, N	9×10^{-8}		4×10^{-8}		5×10^{-7}	
		S		5×10^{-8}				
		C				4×10^{-11}		3×10^{-9}
Na	3.2×10^{-3}	F, N	10^{-10}		3×10^{-10}			
		P, W	10^{-12}					
		C				10^{-13}		3×10^{-11}
		S		10^{-8}				
Ni	5×10^{-8}	F, N	4×10^{-8}		8×10^{-9}		5×10^{-8}	
		P, N	6×10^{-9}					
		C				10^{-11}		5×10^{-12}
Pb	5×10^{-8} [c]	F, N	4×10^{-7}		2×10^{-8}		5×10^{-7}	
		P, N	8×10^{-9}					
		F, Sb				5×10^{-10}		
		S		5×10^{-9}				
		C				5×10^{-12}		10^{-11}

(*Continued*)

TABLE 2 (*Continued*)

Element	Serum conc. (g/ml)	Method[a]	Detection limits[b] Emission g/ml	Emission g	Absorption g/ml	Absorption g	Fluorescence g/ml	Fluorescence g
Se	5×10^{-8} [c]	F, N			5×10^{-7}		3×10^{-7}	
		P, N	2×10^{-6}					
		F, Sb				10^{-8}		
		S		10^{-6}				
		C				10^{-10}		
Si	5×10^{-6}	F, N	10^{-5}		3×10^{-7}		5×10^{-7}	
		P, N		10^{-8}				
		C						3×10^{-9}
Sr	6×10^{-8}	F, N	10^{-9}		4×10^{-9}		3×10^{-8}	
		C				5×10^{-12}		
		P, N	2×10^{-11}					
		S		5×10^{-8}				
V	2×10^{-8} [c]	F, N			10^{-7}		5×10^{-8}	
		C				10^{-10}		6×10^{-10}
Zn	1.2×10^{-6}	F, N	10^{-4}		2×10^{-9}		10^{-10}	
		P, N	5×10^{-8}					
		F, Sb				10^{-11}		
		S		2×10^{-7}				
		C				10^{-14}		2×10^{-14}

[a] F, Flame atomization; P, plasma atomization; S, spark excitation, sample volume ~50 μl; N, nebulized sample, minimum volume ~1.0 ml; C, carbon furnace or rod, sample volume ~10 μl; Sb, sampling boat, sample volume 0.01–1.0 ml; W, platinum or iridium wire, sample volume 0.005–5.0 μl; R, mercury salts in solution are reduced to the metal and the metal vapor carried to the absorption cell by a stream of gas.

[b] Detection limits are quoted in terms of the solution presented to the instrument.

[c] This concentration is an estimate based on the range of values found in the literature.

The method of preparing the sample for assay depends upon the concentration of the element, the sensitivity of the spectrochemical procedure, and any interference phenomena arising from other constituents of the sample. In flame photometry, the sample is usually an aqueous solution, but in other methods it may be necessary to dry the sample on some form of support. With trace elements or micro samples, great care is needed to avoid contamination during sample collection and preparation. Frequently, elements are bound to protein or other organic matrices. As spectrochemical methods measure total element, sample preparation must be used to separate the metal into its various fractions.

Interference in analytical atomic spectroscopy can be severe and many of the advances in instrumentation are directed at minimizing this. The three principal areas where interference can arise are sampling, atomization, and signal processing. With solutions, homogeneity is not a problem, but when dried or ashed samples are placed on an electrode, the more volatile elements will atomize first during the "burn," so that the integrated emission must be measured. The atomization stage includes transport of the sample from the preparation vessel to the vaporization "cell" where atomization and excitation occur. With a solution, changes in viscosity and surface tension affect the supply of sample to the nebulizer. The chemical composition of the dried sample determines the rate at which molecular and atomic vapors are formed on heating, while the composition and temperature of the atmosphere in the "cell" determines the free atom population and the number of excited atoms. Signal processing is generally in two parts: optical, where the spectral line is isolated, and electrical, where its intensity is measured and corrections are made for background emission and other system instabilities. The generation of a signal from a sample is a complex process with many potential sources of error, so it is essential that instruments are operated in a reproducible manner for reliable results to be obtained.

The standardization of spectrochemical procedures is difficult owing to the variety of material analyzed and the impossibility of producing a standard identical in composition to the sample. It is therefore necessary to determine the extent of interfering effects and to use a standard which will compensate for these. However, CV of better than 3% can readily be achieved with accuracies of the same order at the 0.1 μg/ml or 10^{-9} g level. Spectrochemical methods of analysis are accurate and rapid when correctly used, but as the equipment becomes more complex, the operating procedure, quality control and the maintenance necessary to ensure reliability also becomes more elaborate.

All instruments for spectrochemical analysis consist of three parts: a means for vaporizing the sample and exciting atomic emission, some form

of monochromation to select the analytical spectral line, and a photodetector with its associated measuring system. For absorption and fluorescence work a light source must be added. Developments in instrumentation consist of improvements in one or more of these parts.

3.2. Light Sources

Light sources may generate either a continuous or a line spectrum. In atomic absorption little use has been made of the continuous source for two reasons: first, an expensive, high resolution monochromator ($\lambda/\delta\lambda$ = 500,000) is required if the sensitivity is to be comparable with that obtained using a line source; and, second, the energy available within the bandwidth of the absorption process is small. One continuous light source can be used for a number of elements where high sensitivities are not required (F3). The continuous source has also been used to provide background corrections in the analysis of elements with resonance lines in the ultraviolet region where flame gas absorption reduces the precision and sensitivity of the analysis (K20). At least two commercial companies use a deuterium arc to provide a background correction system. In atomic fluorescence a continuous source can be used, as the absorption process itself provides the required monochromation. However, the high intensity sources necessary to produce significant fluorescence can lead to serious problems of scattered radiation unless some form of primary monochromation is included to reduce unwanted radiation. Light sources used in fluorescence include the high pressure pulsed xenon arc (K15) and a 500-W xenon continuum (C25).

Line sources are mostly used in atomic absorption and fluorescence because the energy which can be generated within the bandwidth of the absorption line is much higher than for any continuous source. In the ideal source only the resonance lines of the elements to be analyzed would be excited and these would be narrow, intense, highly stable and capable of selective modulation. Developments in sources have been directed toward this ideal and increasing the range of elements. These improvements will be of greatest benefit in trace-element analysis.

The hollow cathode lamp is the commonest line source used and its construction and operating conditions may be modified to obtain high intensity of the resonance lines (L15). Commercial metal vapor lamps have been used for the alkali metals and zinc, cadmium, tellurium, and mercury. An alternative source of great brightness is the electrodeless discharge tube which uses a silica tube containing a volatile element or compound. Excitation is achieved by placing the lamp in a resonant microwave cavity. At present there is difficulty in obtaining a stable light output, but this problem is likely to be overcome in the near future

(S11). Both hollow cathode lamps and discharge tubes usually contain single elements though certain combinations of element are possible. Instead of using a lamp, an intense emission spectrum may be generated by introducing relatively large amounts of the elements to be measured into an auxiliary flame or arc (R4, S20), which is at a higher temperature than the analytical atomic vapor. These light sources are of limited value owing to poor sensitivity and instability, but they can be useful when the appropriate lamp is not available.

In future the tunable laser may be a practical light source. The high intensity of its radiation could be particularly valuable in atomic fluorescence and preliminary experiments have demonstrated the feasibility of the technique for Ba and Na (F9).

There is a demand for light sources suitable for multielement absorption and fluorescence analysis. So far, seven have been successfully combined within one lamp (F6). Alternatively the emission from a number of lamps (M10, M12) can be combined optically so that failure of one lamp does not interfere with the analysis for other elements.

3.3. Generation of the Analytical Signal

The conversion of the sample into an atomic vapor and its subsequent excitation are the most important and difficult stages in atomic spectroscopy. The process consists of three distinct phases: presentation of the sample to the energy source, atomization, and finally excitation of the atomic vapor. The ideal system is one in which the sample is completely converted into an atomic vapor in a perfectly reproducible manner, the vapor produced is of high atomic density with no interactions within the vapor which could lead to impairment of the emission, absorption, or fluorescence.

3.3.1. *Sample Presentation*

This may be either a continuous process, used when the sample size is relatively large (1 ml or more), or a discrete process, used with samples of less than 20 μl. Continuous-flow systems are simpler to use and more precise, but they are less sensitive. They employ a nebulizer in association with a flame or gas plasma, and either a rotating electrode (Rotrode) or drip-feed to the electrode with the arc or spark. The pneumatic nebulizer has an efficiency of 5–10% and generates an inhomogeneous aerosol. Efficiency can be improved by proper design of the nebulizer and spray chamber (N4), by use of heated nebulizer gas (R5) or ultrasonic devices (S23). The maximum improvement is a 5- to 10-fold increase in sensitivity. There is also an increase in the complexity and cost of the instrument which usually offsets these benefits. The effect

of background instability may be reduced by modulating the sample flow to the nebulizer (M23).

Discrete systems require accurate measurement of small sample volumes onto a platinum or iridium wire loop (V6), copper or graphite electrodes (N1), nickel or tantalum boats (D12, K2), or into a graphite furnace (M7). After drying *in situ* and possibly ashing, the sample is thermally atomized by a flame, electrical current, or arc. Electrolytic deposition onto an iridium wire followed by flame atomization into a long-tube atomic absorption system has been used to measure ionic Cu in blood plasma (E2). The CV of these methods is about 5%; when greater precision is required, replicate analyses are usually possible owing to the limited volume of sample required. Solids may be analyzed directly by mixing with powdered graphite and packing a hollow electrode with the mixture or by briquetting the powder.

3.3.2. *Atomization*

Atomization should completely convert the elements in the sample into an atomic vapor of high density. To meet these requirements a large amount of energy is injected rapidly into the sample; hence, arcs, sparks, high temperature flames and lasers are used for this purpose. The shape of the atomic cloud generated is determined by thermal expansion of the vapor and the flow of inert or flame gases. This system forms a dynamic atom cell or reservoir.

At present the flame is the most commonly used energy source for atomization in clinical chemistry. The principal gas mixtures used are air/town gas or propane (2100K), air/acetylene (2500K), and nitrous oxide/acetylene (3000K). The rate at which the desolvated sample vaporizes either in an aerosol or from a solid support depends on its chemical composition and the flame temperature. High temperature flames give rapid and complete vaporization (W21), but require greater care, as their higher burning velocities make them more susceptible to flashback. Hotter flames result in greater expansion of the flame gases, and hence a reduction in the atomic vapor density. Once the sample is atomized the elements can react with the flame gases and sensitivity is reduced if stable compounds are formed. A fuel-rich flame minimizes oxide formation and the nitrous oxide flame is very satisfactory for the analysis of refractory materials owing to its high temperature and chemical composition (C14). Most interferences in flame photometry arise in the atomization process due to differences in composition between the sample and standard. The use of solutions as dilute as possible generally reduces interference.

Although flame methods will be dominant in clinical applications of

atomic spectroscopy for some time to come, their potential is nearly fully exploited due to the limits on design imposed by flame temperature and the burning velocity. Electrical methods do not suffer from these limitations and may be expected to develop further, although their flexibility leads to lower precision unless stringent controls are applied. There are two types of electrical atomizer: first, discharge devices where the sample is either burnt off from an electrode (N1) or injected into a gas plasma as an aerosol (D15), and, second, devices where a current is passed through the sample support, iridium wire (V6) or graphite tube or rod (M7) to vaporize the sample. The latter type generates very little background signal and is therefore particularly useful for atomic fluorescence. Plasmas may be generated by dc (V1), rf (D15), or microwave, while electrically heated furnaces use dc, ac, and rf (M22) to provide the power. Discharge systems are used principally in emission analysis, and furnaces are used for absorption and fluorescence.

Many devices have been developed to meet special needs. Pulse atomization, by focusing a laser beam through a microscope onto the target, offers a means for measuring the metal content of individual cells (T11). Electron bombardment has also been used (R15) for atomization of micro samples. Analyses have been made on 10 nl of body fluid using the helium glow photometer (V6), in which the sample is vaporized in a sealed chamber by electrical heating of the Ir support wire and subsequently excited by rf discharge in the He atmosphere. Mercury can be released from aqueous solution by reduction with stannous chloride or hydrazine and the metal is then atomized into a cell by passing a stream of air through the solution; 1 ng can be detected (T5).

3.3.3. *Excitation, Absorption, and Fluorescence*

Once the atomic vapor has been generated, it should be observed in the atom reservoir for as long as possible. This reservoir is the part of the flame viewed by a monochromator, the cathode layer of an arc, or the cuvette of a spectrophotometer. In many systems the residence time is of the order of 1 ms, but for absorption purposes it may be increased 10–100 times by passing the vapor down a tube (A10). The reservoir itself should be as free as possible from background emission or absorption. In flame methods some reduction of background emission can be effected by shielding the flame with an inert gas to prevent the entrainment of room air, thus eliminating the secondary reaction zone from the reservoir (H18). In emission analysis the reservoir should be as thin as possible in the viewing direction to minimize self-absorption, while for absorption work it should be as long as possible.

When the element flows continuously into the excitation zone and the

signal is observed for 5 seconds or more, the detection limit for flame, arc, or plasma corresponds to an element flow rate of approximately 10^{-10} g s^{-1}. In a transient response system, where vaporization takes place in less than 1 second, the flow rate may be as low at 10^{-12} g s^{-1}.

Once atomization is complete, temperature is important only in emission analysis. To minimize background emission the temperature should be as low as possible compatible with generating an adequate analytical signal. Promising developments in emission analysis are the rf and microwave plasmas. In these systems the sample is nebulized with argon and the aerosol is desolvated and fed into an argon plasma. The high temperature of the plasma dissociates and excites the sample in an inert atmosphere. Outside the region of the plasma (the "tail flame") the gas temperature is about 3000K and there is little background emission from the carrier gas. Detection limits are low (ng/ml) for many elements (D15). The excitation temperature (approximately 8000K) of arcs, sparks, and plasmas is comparable with, or greater than, that of the discharge lamps used in absorption and fluorescence analysis. The detection limits by emission will therefore be lower than can be achieved using the same atomic vapor for absorption analysis.

As an emission signal is very dependent upon the excitation temperature, which in turn is determined by complex interactions within the source, a CV less than 0.5% is difficult to obtain and the sensitivity will vary from one occasion to another. The effect of this is reduced by the use of an internal standard element, but this is of little value in absorption and fluorescence unless it also serves as a releasing agent in the atomization process.

3.4. Wavelength Selection

The function of the spectrometer is to accept as much light from the source as possible and to isolate the required spectral lines. This may be impossible where there is a continuous spectrum in the same region as the analytical line; for example, the magnesium line of 285.2 nm coincides with a hydroxyl band. In direct reading instruments, electronic devices may be used to supplement the resolution of the spectrometer by modulating the intensity of the analytical signal. In absorption and fluorescence the light source is modulated; in emission the spectral line is scanned (S16) or the sample flow modulated (M23).

In atomic absorption the background continuum is usually negligible and the resonance line intense. To give the maximum discrimination against stray radiation, and hence the lowest detection limit, the slit width should be small. In atomic emission and fluorescence the analytical signal is smaller and the background due to scattered light and con-

tinuum of the flame is relatively large. The slit width should therefore be as wide as possible, commensurate with eliminating spurious signals from the sample, thus giving the maximum signal and highest precision of measurement.

As the light collection capacity of a dispersive monochromator is frequently low, the use of filters can lead to more precise measurements of emission signals if the bandwidth is sufficiently narrow to avoid spectral interference. Interference filters with a bandwidth of 5 nm are available, and for maximum selectivity these should be used with near parallel light (L7). In atomic absorption the light collection capacity of the monochromator is frequently unimportant as the source intensity is high and the cross section of the optimum absorption zone of the flame is small.

Multielement analysis may be made either by measuring each element in turn using a single-channel instrument or by simultaneous observation of the emission, absorption, or fluorescence of all the elements of interest. The multichannel instrument may observe all analytical lines simultaneously or in rapid sequence. Sequential methods, using either filters, scanning mirrors, or gratings, lead to a cheaper instrument but there is a loss of information as each element is measured only for a fraction of the total exposure time. As the sample excitation conditions may be time-dependent, systematic errors can arise unless the scanning rate is rapid. Simultaneous multichannel instruments use a photographic plate, an array of photomultipliers, photodiodes, or a television camera tube as radiation detectors.

High resolution and large apertures can be obtained with interferometers. An echelle grating with these qualities has been used in flame photometry (C24), but generally these devices have not been widely used except in the greatly simplified form of interference filters. With the development of Fourier transform spectroscopy (H21, M11) multielement analysis using interferometry may be possible in the future. Though this is a scanning technique, any part of the spectrum can be observed for almost 50% of the time. The signal generated in the photodetector is encoded so that spectral line intensities may be derived by computer analysis. A simpler form is known as Hadamard transform spectroscopy (D9). This technique can be used in a conventional spectrograph by fitting a movable slotted mask in the focal plane. After passage through the mask the spectrum is reflected back through the instrument to fall on a photomultiplier near the entrance slit. The output of the photomultiplier arises from a combination of wavelengths, and the contribution of any one wavelength varies as the mask moves. By relating the photomultiplier signal to the corresponding mask position,

a series of simultaneous equations can be set up from which the intensity of the required spectral line can be computed.

The stability of the wavelength setting of a monochromator can be a problem in high resolution spectrometry. This difficulty has been overcome by the use of the resonance monochromator (S24), consisting of a hollow cathode lamp modified to produce only an atomic vapor. The vapor is irradiated with the light to be analyzed and fluorescence occurs at the resonant wavelength of the cathode element. The intensity of the fluorescence is proportional to the component of that wavelength in the primary radiation.

Various optical devices provide double-beam facilities and arrangements for monitoring background absorption, and others provide long absorption paths and improved light-gathering power for emission and fluorescence analysis. Although these devices give improved performance in particular situations, they are not widely used as they frequently are an additional expense and complication.

3.5. Detectors and Measuring Systems

Photographic recording of a spectrum is rarely used in clinical chemistry owing to its poor precision and the inconvenience of processing and densitometric measurements. However, it has advantages: (1) all spectral lines emitted by the sample within the range of the instrument are recorded, and hence unexpected elements may be observed; (2) as the signal is integrated for the whole of the exposure period, the effect of short-term fluctuation in emission intensity is eliminated. Computers have been used to improve the densitometry of the photographic plate.

More commonly, photoelectric devices are used to measure directly the intensity of the spectral lines. Solid state detectors such as barrier layer cells are well established for the measurement of relatively high intensities in the visible region of the spectrum (see Section 4.4). Newer detectors include semi-integrated devices whose output signal is a series of pulses, with a frequency proportional to the light intensity, or arrays of photodiodes for multielement analysis (B11). At medium light intensities, vacuum photocells or photomultipliers are preferred for their sensitivity and stability (see Section 4.4). Their spectral response ranges from approximately 180 nm to 800 nm with a peak quantum efficiency of the order of 15% in the blue region. One type of photomultiplier with no response at wavelengths greater than 310 nm ("solar blind") can be used in some absorption and fluorescence analyses without further monochromation (L3).

The output signal of a photomultiplier is essentially digital, with a shower of electrons resulting from the receipt of each photon. If a low

light intensity is used, it is possible to resolve the individual pulses and the frequency of these is a measure of the rate at which photons strike the photocathode. This technique of photon counting can be used with conventional photomultipliers and applied to all types of spectrophotometry (D5, F8), giving greater sensitivity to low light levels, accurate signal integration, and improved precision. Since very narrow slits can be used, spectral resolution is improved. The performance of photomultipliers at low light levels is further improved by cooling, which reduces dark current without significant effect on light sensitivity.

The photodetector output usually requires amplification and, if the signal has been modulated to eliminate the background and reduce noise, it must now be decoded. The system for this may be simply a tuned circuit or, more elaborately, phase sensitive or lock-in detectors. If the resultant signal is not proportional to the element concentration it may be linearized with simple analog circuits.

Currently, instruments from several manufacturers have built-in facilities to sample the signal for adjustable periods or alternatively, the sampling time is defined but the number of periods may be varied, the answer being presented as the mean signal for the number of periods employed. This provision gives the analyst some control over the precision of the analysis. For example, if the signal is strong, sufficient information may be collected in a single observation period; conversely, if weak, many integration periods may be appropriate. The maximum observation period is determined by the long-term stability of the instrumental zero and sensitivity.

3.6. Conclusions

The use of analytical atomic spectroscopy in clinical chemistry has developed rapidly over the last 20 years and there is now adequate knowledge and instrumentation available for the measurement of a wide range of elements (C12, H25, M4, W25) in concentrations as low as 1 ng/ml or amounts as small as 10^{-12} g. The cost of the instruments ranges from £100 ($240) for the simplest flame photometer to £50,000 ($120,000) for an advanced direct reading spectrometer with data handling facilities.

A simple emission flame photometer is adequate for Na and K while a more selective emission/absorbance system is necessary for Ca, Mg, and trace metals. The range of trace metals which can be analyzed (e.g., Cu, Zn, Fe, As, Pb, Co, Mo, Se, Cd, Hg) with an instrument depends on the efficiency of atomization, excitation, and light collection, as well as the intensity and stability of the background. Owing to the difficulty of obtaining complete stability of baseline and sensitivity, frequent standardization of instruments is usually necessary. This can

make an analysis difficult to automate beyond the stage of mechanically presenting the sample to the instrument.

4. Ultraviolet and Visible Spectrophotometers

Photoelectric colorimeters and spectrophotometers are used to measure the absorption by solutions of electromagnetic radiation in the 200–800 nm range. The wavelength absorbed is characteristic of an electronic transition within the absorbing species. The width of the absorption band (0.5–50 nm) is much greater than that of an atomic line, due to the fine structure of the energy levels arising from molecular vibration and rotation. The wavelength and intensity of the band may be affected by the solvent and other components of the solution. The absorption spectrum of a compound or its colored derivative provides a rapid and sensitive method for qualitative and quantitative analysis. In a complex mixture the width of absorption bands frequently results in band overlap. The principal limitations are set by the chemical nature of the sample, and many developments are directed at improving the specificity and sensitivity of the reagents used to develop colored compounds.

The basic components of spectrophotometers are a light source, wavelength selector, absorption cell (cuvette), and photodetector. Colorimeters or absorptiometers commonly use nondispersive wavelength selection (a filter with bandwidth 4–40 nm) and solid state or simple phototube detectors, while spectrophotometers employ a prism or grating monochromator (with bandwidth down to 0.2 nm) and a photomultiplier. Colorimeters are inexpensive and most appropriate for repetitive measurements of absorption at a fixed wavelength. The more expensive spectrophotometer can also fulfill this function, but its main purpose, by virtue of its accurate and variable wavelength control, is the measurement of absorption spectra.

4.1. Light Sources

A spectrophotometer requires a highly stable source emitting an intense continuous spectrum. No single source is suitable for the complete ultraviolet and visible range. Most instruments use a tungsten filament lamp for wavelengths greater than 350 nm and a hydrogen or deuterium discharge lamp for shorter wavelengths.

Earlier types of tungsten filament lamp showed little or no emission at wavelengths below 350 nm. "Overrunning" the lamp by operating at a higher temperature tends to increase the volatilization of tungsten, which is deposited as a metallic mirror on the inside surface of the lamp, thus reducing its output. The addition of iodine prevents this effect, by forming volatile tungsten iodide, which, on contact with the

hot filament, redeposits the tungsten and iodine reenters the cycle. With sensitive photodetection, a 1-kW quartz–iodine lamp can be used as a standard light source over the range 250–2600 nm (K19).

Gas discharge lamps are suitable sources of ultraviolet (UV) radiation. Colorimeters, with mercury lamps and appropriate filters, are being increasingly used for multiple measurements of UV absorbing compounds eluted from chromatography columns (T2). The emission spectrum of a hydrogen gas discharge arises from electron transition between a large number of overlapping energy levels, resulting in a quasicontinuous energy spectrum, increasing in intensity in the ultraviolet, but falling off rapidly above 350 nm. Deuterium lamps show a similar spectrum with approximately three times the radiant output of the hydrogen lamp (E1). Both show characteristic lines at 656.3, 486.1, and (weaker) 379.9 nm, and with a high grade silica envelope emission down to 160 nm can be obtained. The extra-high-tension xenon lamp gives an intense continuum, but requires water-cooling. An alternative high intensity source is the dc argon arc (A14). Both these sources are unnecessarily powerful for most uses, but if a double monochromator is used a high intensity source may be necessary to obtain sufficient energy in the emergent beam.

The carbon lamp is an intense source of monochromatic UV (193.1 nm) radiation (W4). Other monochromatic light sources can be constructed for a limited range of wavelengths by exciting the resonance emission of an atomic vapor (S24). These have a stable wavelength but the emission is unstable. Lasers provide high intensity monochromatic radiation for a number of wavelengths but they are of limited value in absorption spectrophotometry.

4.2. Wavelength Selection and Optics

When absorbance is measured, the bandwidth of the light passed by the wavelength selector must be small compared with that of the absorption band. If this condition is not fulfilled, the observed absorbance will be less than the correct value owing to the contribution of unabsorbed radiation to the total signal. In addition the wavelength of maximum absorption may be displaced from its true value.

Most colorimeters now use interference filters, whose peak transmittance can be adjusted during manufacture to almost any desired wavelength, or an interference wedge covering the range 400–700 nm (P3). Gradual changes in the transmission of interference filters have been found when high intensity light sources were used (H11). With all filters, narrower bandwidths result in lower transmission and hence low signals. The selection of a filter is therefore a compromise between

bandwidth and signal intensity. Many medium-priced instruments use diffraction gratings with bandwidths down to 4 nm; this may not be sufficiently narrow to measure the true absorbance of compounds with narrow bands. The stability of colorimeters has been improved by the use of a "two wavelength" technique, in which the transmission of the sample is measured alternately at the absorption maximum and at a nearby unabsorbed wavelength by alternate interposition of two filters. The ratio or difference between these two signals is computed electrically. By this means any drift in the intensity of the light source or in photodetector sensitivity is corrected. An ingenious variation of this technique for use in the UV alternately interposes a filter of fluorescent material between the source and sample and then between the sample and detector. The detector measures the change in fluorescent signal (R12).

Most dispersive monochromators (prisms or gratings) are of conventional design, which has been unchanged for some years. The requirements of a monochromator are accurate wavelength calibration, good light transmittance, and adequate resolution ($\lambda/\delta\lambda \sim 5000$). The choice of slit width is often a compromise between a wide slit, which gives more light and therefore greater photoelectric response and sensitivity, and a narrow slit giving greater spectral purity and less stray light but requiring high electronic sensitivity (gain) leading to more noise. The light transmission of a prism is greater than that of a grating, but owing to the nonlinear dispersion of glass and quartz prisms, measurements can be made only at constant bandwidth if the slit width is adjusted for each wavelength. A sapphire prism has been used for measurements in the 180–200 nm range (T7). Where "blazed" gratings are used to improve light transmission efficiency in a wide range instrument (180–800 nm), a mid-scale changeover of gratings may be required. A nitrogen purge may be used to improve ultraviolet transmittance, and thermostatting improves wavelength stability.

In the double beam or ratio recording spectrophotometer, light from a single source is reflected alternately through sample and reference cuvettes by a reciprocating mirror or by passing through a beam splitter and vibrating shutter. The modulated beam falls on a photomultiplier, the output of which is amplified and decoded before being passed either directly to a potentiometric recorder or to a servomotor which moves an attenuator to balance the intensities of the two beams, this movement being linked to the recorder pen. The instrument measures the ratio of intensity of the two beams and readings are independent of fluctuations in the light source and are also corrected for any absorp-

tion by the solvent in the reference cuvette. With a suitable wavelength drive, the ratio can be continuously recorded over the spectrum in transmission units or, after logarithmic transformation, in absorbance. When two photodetectors are used, the electrical processing of the signal can be simplified as no decoding is necessary, but matching of the photodetectors is then a problem. One application of the double beam instrument is in kinetic enzyme assay (T10). Duplicate reaction mixtures, initiated a finite time (Δt) apart, are placed in the two beams. With zero-order kinetics, the difference in absorbance (ΔA) is constant and the rate is given by a single measurement ($\Delta A/\Delta t$).

Modifications to dispersive monochromators include double monochromation and dual wavelength systems. The object of double monochromation is to give increased discrimination between radiation of the required wavelength and background signals arising from scattered light. Radiation is either passed through two monochromators in series or through the same instrument twice (H3). Dual-wavelength systems employ two diffraction gratings mounted one above the other (S7) to measure simultaneously the absorption at two adjacent wavelengths. When the instrument is operated in the scanning mode, the difference in absorption (dA) can be used to plot a derivative spectrogram ($dA/d\lambda$ vs λ). This method of data presentation enhances the visibility of fine detail in a conventional spectrogram and leads to lower detection limits (K16). In the static mode, if the spectrum is simple and the wavelength separation ($d\lambda$) relatively large, the instrument can be used as a double beam spectrophotometer where one channel monitors the intensity of the light source and the other the absorption maximum. High speed, narrow range spectral scanning by means of a quartz plate (S16) or an electrooptical material (W5) can be used in conjunction with conventional slow scan to generate a derivative spectrogram using a single diffraction grating. Quantitative analysis of a known multicomponent mixture can be carried out by measurement at a few preselected wavelengths, using a programmable spectrometer. Wavelength programming is still in its early stages and would be particularly useful for repetitive measurements of absorbance at several specified wavelengths, as, for example, with the Allen correction for nonspecific absorbance in steroid assays.

Large aperture, high resolution spectrometry can be achieved using interferometers. A scanning system used in conjunction with Fourier transform spectroscopy (see Section 3.4) would facilitate the rapid measurement of complex spectra. As computer facilities are essential, the technique lends itself to automated analysis.

4.3. Cuvettes

A spectrophotometer is no better than its cuvettes (E1), and an immense variety is available. In colorimeters cylindrical cells give results as good as those obtained with rectangular ones (B20), but in spectrophotometers, curved surfaces lead to internal reflection with consequent loss of radiant energy and errors from stray light. Tube holders have been devised to reduce these errors (D4, H5). Microcells (5–500 μl capacity) are designed to give the maximum optical pathlength with the minimum solution volume (K12). Devices have been described for eliminating gas bubbles from flow cells and for increasing their effective length by multiple internal reflection. For static observations, variable pathlength cells have been used to give more precise measurement of absorbance (A4). Special cells have been described for use at low temperature (W27) and for spectrophotometric titrations (A13). For strongly absorbing solutions, thin cells have been developed (M21) and multiple pass cells for weakly absorbing samples (G8). Absorbance of some compounds varies with temperature, so temperature control of cuvettes is useful.

The material from which the cuvette is made determines the wavelength over which it can be used. One specification (B14) states that rectangular cuvettes with pathlengths between 1 and 40 mm should have a tolerance of pathlength of 0.02 mm for silica cells and 0.5% or 0.02 mm (whichever is the greater) for glass cells. Accurate spectrophotometric work requires matched cells and a pair which is optically matched at one wavelength may not be matched at another. Maintenance and cleaning of cells are important factors in obtaining good results, but these are problems of technique rather than instrumentation (B21).

4.4. Detectors and Output

The three basic types are photoconductive, photovoltaic, and photoemissive, and all are sensitive to both heat and light. The resistance of a photoconductive cell is lowered when it is illuminated and, over a small range, its response is linear. Cells containing lead sulfide, which is sensitive at wavelengths greater than 700 nm, and cadmium sulfide or selenide, with a sharp response maximum at 710 nm, have been used but may not give a stable response and are largely restricted to specialized applications in other fields. Silicon photodiodes and transistors are sensitive from 340 to 1200 nm with a peak at 900 nm.

A photovoltaic cell generates a current which may be registered by a galvanometer without amplification. The commonest type is the barrier

layer selenium photocell, which is cheap and rugged, but usually limited to 400–650 nm, although its range and sensitivity can be extended by sensitization, possibly into the ultraviolet (E1). With a low external resistance, the photoelectric current is proportional to light intensity but can be made proportional to its logarithm by selection of a suitable high resistance, thus giving a linear absorbance scale. Both the dark current and sensitivity of photovoltaic and photoconductive cells are dependent on temperature. This can be overcome by means of compensating circuitry, but the cost then becomes comparable with that of the vacuum photocell, which does not suffer to the same extent from these difficulties.

A photoemissive cell is a vacuum tube containing two electrodes: a cathode coated with an alkaline metal or alloy which emits electrons on exposure to light, and an anode, consisting of a grid of fine wire held at 60–150 V positive with respect to the cathode. Blue sensitive phototubes usually employ Sb–Cs-coated photocathodes in a silica envelope and respond to light from about 180 to 600 nm, whereas the red-sensitive type, used above 600 nm, contains cesium oxide. Wide-range spectral response is obtained using composite photocathodes (e.g., Ag–Cs and Sb–Cs). Phototubes are used when light intensities are relatively high. A photomultiplier is a phototube with internal amplification of the photocurrent. Each photoemitted electron is accelerated by an applied potential to strike another electrode (dynode) where it releases a further 4–5 electrons as secondary emission. Several such dynodes set up a chain reaction giving an amplification of about 10^6. Most spectrophotometers use photomultipliers; response is rectilinear under normal illumination, and they have high sensitivity, short response time, and little fatigue. The use of photomultipliers for photon counting has been outlined in Section 3.5. The design and operation of a double-beam photon counting photometer has been described by Ash and Piepmeier (A11). Cooling of photomultipliers reduces the dark current. Liquid nitrogen is used for this purpose, and the evaporating gas can be used to purge the monochromator (C6). As the sensitivity of a photocathode varies over its surface (B8), care must be taken to ensure either that the same part is used for all measurements (e.g., in a temporally modulated double-beam and dual-wavelength system) or that all the photocathode is illuminated. The response of a photodetector which is sensitive only in the visible range can be extended into the ultraviolet by the use of fluorescent materials (phosphors).

Absorbance may be derived from the photoelectric signal either directly on a precalibrated meter scale or by a null method where a reference signal is provided optically or electrically. In null methods, readings

may be made logarithmic by using a logarithmic potentiometer or attenuating wedge. Alternatively the output of a photodetector may be converted into a logarithmic function of light intensity by appropriate circuitry.

Digital display is expensive but may be justified by reduced transcription errors, greater speed, and compatibility with data processing systems, printers, tape punches, and computers. In some systems, the output is fed directly to an analog-to-digital converter and then to the computer. These systems have been used for repeated single or multicomponent analysis (L9) or kinetic studies (T8). In wavelength scanning, both the wavelength setting and the output of the spectrophotometer must be simultaneously digitized.

4.5. Errors

It is widely assumed that wavelength accuracy, photometric linearity, and photometric accuracy are inherent properties of spectrophotometers. However, several trials (R1) have shown that the absorbance and wavelength of absorption maxima of the same solution measured on different instruments can vary widely. A major source of error is stray light emerging from the exit slit together with the chosen region of the spectrum. The tungsten lamp has intense emission in the visible but is relatively weak in the near ultraviolet, so that in work at wavelengths below 450 nm, some light of longer wavelengths may be reflected back through the exit slit. The hydrogen lamp has relatively weak emission in the far ultraviolet, and stray light from its more intense wavelengths can give spurious readings so that instruments are rarely usable below 200 nm unless the stray light is substantially reduced. Stray light may be reduced to negligible levels by double monochromators or insertion of appropriate filters (E1, R1), and in some instruments this is done automatically at preselected wavelengths. Errors are usually manifest as false absorption maxima, low absorbance readings, and nonlinearity of calibration curves. According to Edisbury (E1), stray light is more often due to neglect, for example to dust, than to bad instrumental design, so frequent checks and good maintenance are essential.

Wavelength accuracy can be checked by locating deuterium or mercury emission lines or using didymium and holmium oxide glasses. In an instrument with a prism any three correct wavelengths should guarantee the accuracy of all others, but with a grating instrument two correct readings should be sufficient (E1).

Photometric linearity is usually tested with colored solutions of varying concentration (B20, R1), but apparent deviations from Beer's law do not distinguish between the properties of the solution (S6) and true

photometric nonlinearity. A narrower spectral bandwidth will often give more linear curves. Reule (R7) showed that the Beer-Lambert test was inadequate for assessing the linearity of reference instruments and described a light addition method which would reveal nonlinearity of less than 0.1% in individual components. Many instruments claim linearity of ±0.001 absorbance unit.

Accurate measurement of absorbance is unnecessary with colorimeters as readings are usually compared with those of standards. Molar absorbances measured with a spectrophotometer are often used for calculating concentration and absorbance accuracy is then essential. Most methods of checking absorbance accuracy (E1, R1) depend on the use of calibrated glass filters or standard solutions. Filters such as holmium oxide and didymium may fade and require careful location in the instrument. Solutions such as potassium dichromate seem preferable as they are easily prepared and simple to use. According to Rand (R1), no photometric measurement can be considered reliable unless the instrument has been checked with suitable standards. Although there is currently no agreement on the "true" absorbance value of solutions, Rand (R1) concluded that a 0.05 g/liter solution of high purity potassium dichromate in 0.01 *N* sulfuric acid has an absorptivity of 10.70 at 350 nm with an uncertainty of less than 0.3%.

4.6. Conclusions

Present-day colorimeters and spectrophotometers show little resemblance to their predecessors of 25 years ago. As independent instruments they now seem to be at the limit of their development, but they are increasingly incorporated into automatic analytical systems and as monitors for chromatography columns. New developments and components (B9, C27) give better spectral resolution and sensitivity and allow some degree of automatic programming and control, but in clinical chemistry the more complex instruments are likely to be in competition with alternative methods such as GLC, NMR, and mass spectrometry.

5. Fluorimeters and Phosphorimeters

A molecule may re-emit absorbed energy as fluorescence radiation within 10^{-3} to 10^{-8} second, or as phosphorescence which occurs after 10^{-3} second or more. These processes can take place in molecules in gaseous, liquid, and solid phases, although not necessarily in all three phases of the same substance. Deactivation or quenching of the excited molecule can occur through radiationless processes such as collision with the walls of the vessel or with other atoms or molecules; increase in the vibrational and rotational energies of the molecule; or, in solids, transfer

of energy to the matrix. Phosphorescence emission can be observed only when the substance is dissolved in a rigid medium, for otherwise the extended lifetime makes quenching likely to occur before radiation is emitted. The three fundamental parameters in fluorescence and phosphorescence are the wavelengths of the exciting and emitted radiation and the time interval between the two processes. Appropriate combinations of these three parameters provide highly specific and sensitive methods of quantitative analysis.

Instruments for luminescence measurements in the spectral range 180–650 nm use the same basic components as spectrophotometers, except that two light beams of different spectral composition are involved. The incident or primary beam is directed at the sample, which absorbs the radiation within its activation spectrum. The excited molecule then emits a characteristic fluorescent spectrum of longer wavelength, and the intensity of this secondary beam is used for quantitative analysis. The amount of luminescence is proportional to the intensity of irradiation and the amount of fluorescent material in the cuvette. The analytical signal is proportional to the luminescence, the light-collecting efficiency of the analyzing monochromator, and the sensitivity of the detector.

A fluorescence spectrometer (or spectrofluorimeter) is provided with two monochromators to study both the excitation and fluorescent spectra. The two spectra are used in the elucidation of structure and identification of the molecule, as well as in defining the optimum conditions for quantitative determination. A fluorimeter uses filters in each beam. For the observation of luminescence decay, shutters are interposed alternately in the primary and secondary beams.

5.1. Light Sources

These are similar to those used in absorption spectrophotometry, but with higher intensities to compensate for light losses in the more complex system. As most excitation spectra lie in the ultraviolet, tungsten filament lamps are rarely used. Mercury lamps may be coated with phosphors which will absorb spectral lines and emit a continuum. A source generating a continuum is necessary to measure excitation/emission spectra. For repetitive determinations of a single component a high intensity line source (metal discharge lamp, arc or hollow cathode lamp) with emission at a peak of the excitation curve may be used, with a simple monochromator (e.g., a filter) to isolate the required wavelength. Lasers are beginning to be used as sources, and their intensity and extreme monochromaticity are potentially useful for weakly fluorescent materials (R8, S8). Microwave discharge lamps, which can

now be made for a wide variety of wavelengths, are well established in atomic fluorescence and may find a place in molecular luminescence work. Higher light intensities can be obtained by pulse operation of discharge lamps (W23). The use of pulsed light sources or fast chopping of steady-light sources (H19) is necessary to obtain luminescence decay curves.

5.2. Wavelength Selection

The primary monochromator selects the excitation wavelength and removes unwanted radiation. The analyzing monochromator selects from the luminescent radiation the required wavelength and discriminates against scattered primary radiation. Monochromators need not be of high resolution ($\lambda/\delta\lambda \approx 5000$) but should be of large aperture.

Glass, gelatin, interference, or liquid filters are commonly used in fluorimeters for repetitive analyses in conjunction with line sources (R16). Although prisms have high light transmission, their nonlinear dispersion is a difficulty when spectrum scanning and therefore gratings are widely used. As spectral scanning is an important part of luminescence analysis, there would be a gain in speed if the emission spectrum could be analyzed by Fourier or Hadamard transform spectroscopy (see Section 3.4).

5.3. Cuvettes

Scattered light and background luminescence from the cuvette can lead to spurious results with low intensity signals. To minimize this error, the angle between the incident and emergent beams is set between 60° and 90°. Only the sample should be irradiated by the source and viewed by the detector. When filling a cuvette, care is necessary to avoid contamination of the sample and surface of the cell and to ensure that the meniscus is outside the optical path. The placing of mirrors in the sample chamber can increase the light collection efficiency of the system, but these additional surfaces may lead to an increased background due to scatter and luminescence light. Glass or quartz cells are commonly used for liquid samples, but when the fluorescence of normal quality materials limits the analysis fluorescent-free quartz cells are available. Luminescence is a temperature dependent process, and so thermostatting of the cuvette is frequently necessary. Cooling to low temperature (e.g., liquid nitrogen) reduces emission bandwidths and increases their intensity, but equipment (L12) for this is not generally available commercially. For the direct examination of thin-layer chromatograms, the TLC plate may be mounted above the sample chamber

and the light beams deflected to excite the fluorescence on the plate as it is driven over the sample chamber.

If a sample is irradiated with polarized light, only those molecules with absorption axes parallel to the plane of polarization will absorb appreciable energy. The emission from the molecule is also polarized, and its plane of polarization will be fixed in relation to its absorption axis. If the molecule has not moved between the absorption and emission processes, all the emitted radiation will be in one plane of polarization. The spread in the plane of polarization of the emitted light is a function of the lifetime of the excited state and the rate of molecular movement. Polarization data give information on molecular size and shape and may be obtained by a combination of spectrum scanning with modulation of the emission signal by rotation of a polarizing film interposed between the sample and detector (K7). Most manufacturers supply a simple, manually operated attachment for polarization studies.

5.4. Detectors

Fluorescence intensities can be expressed in terms of quantum yield, i.e., the ratio of the number of photons emitted to those absorbed; this is a function of the exciting wavelength and temperature. Quinine sulfate, yield about 50%, is commonly used as a standard, but the yield in other materials may be higher, and that of 9,10-diphenylanthracene is reported to be unity (R17).

Photomultipliers are almost universally used as detectors, and more than one may be required to cover a wide range of wavelengths. The light flux falling on the photocathode may be modulated as a result of incorporating facilities to monitor the spectral response of the instrument. This signal is decoded and used to generate excitation and emission spectra which can be automatically presented in quantum or energy units. By this means much closer agreement between the absorption and excitation spectra of molecules has been obtained (C28). Computer calculation using pre-recorded calibration data has been used to effect the same correction (M17).

Luminescence decay curves may be observed by displaying the output of the photomultiplier on an oscilloscope. Precautions must be taken to correct for instrumental distortion of fast decay curves (D13). In multicomponent systems with differing decay times, electronic gating may be used to isolate the signal due to one component (time resolved phosphorimetry) (S1). A complete emission spectrum can be observed using a spectrograph with a photographic plate or television camera tube, but these systems are as yet only of specialist interest.

5.5. Conclusions

The luminescence properties of molecules are widely used for analytical purposes (R16, W12). Sometimes the molecule itself fluoresces, but more commonly, some form of chemical reaction is required to produce a fluorescent compound. With the development of suitable reagents and separation procedures, some forty elements including selenium (W6) and tellurium (K11) have been determined at concentrations of 1 μg/l. Instrumentation has been unchanged in basic design for some time and is adequate for most applications. The multidimensional nature of the technique, involving excitation and emission spectra, polarization, time and temperature dependence, makes prediction of future developments difficult. The majority of instruments used in routine clinical chemistry are likely to remain relatively unchanged apart from provision for automatic sample handling and readout in concentration units. More advanced instruments will be used to obtain high resolution spectra by cooling the sample, by fast response systems, and by more efficient utilization of the emitted radiation. The amount and complexity of the data which can be generated make the widespread use of computers inevitable, and this area may show the greatest development over the next decade.

6. Infrared and Raman Spectroscopy

The infrared region of the electromagnetic spectrum is used for the study of molecular vibrations. For any molecule, the pattern of absorption bands is as unique as a fingerprint, and many individual bands can be related to specific groups or structures. This feature may be used for the identification of a molecule or functional group and for the quantitative analysis of simple mixtures. As infrared spectra are complex, mixtures may need separation before measurements can be made.

Energies in the infrared spectrum are conventionally expressed in wave numbers, which are defined as the number of waves per centimeter, i.e., the reciprocal of the wavelength measured in centimeters. The infrared spectrum extends from 12,500 to 50 cm^{-1} (i.e., a wavelength of 0.8–200 μm) and the far infrared from 40–10 cm^{-1} (250 μm–1 mm), but the upper limit of most commercial instruments is about 200 cm^{-1} (50 μm). Spectra are most frequently obtained by absorption and reflection techniques, but polarization, emission, and luminescence are also used (C26). Similar components are used in all types of instrument. Reflection measurements of samples with low transmission are made in the near infrared with a conventional spectrophotometer fitted with a reflec-

tance attachment. The attenuated total reflection (ATR) technique is based on the observation that if an absorbing substance is placed in close contact with the reflecting face of a prism, the energy which escapes temporarily from the prism is selectively absorbed (G5). Absorption spectra are independent of sample thickness and the method can be used with tissues.

6.1. Radiation Sources

Those most commonly used generate a continuum. The Nernst-Glower is a mixture of zirconium and yttrium oxides heated to a temperature of 1500–2000°C, with a maximum radiation at about 7100 cm^{-1} (1.4 μm). The Globar source is a rod of silicon carbide heated to 1300–1700°C, water cooled, and with a maximum radiation at 5200 cm^{-1} (1.9 μm). A simple source suitable for use over the range 625–3800 cm^{-1} (16–2.6 μm) is the electrically heated nichrome strip. At wave numbers less than 100 cm^{-1}, greater energy is provided by the high pressure mercury lamp. For the quantitative analysis of simple mixtures with well resolved absorption bands, lasers could be suitable sources of monochromatic radiation if tuned to the wavelength of maximum absorption (D2).

6.2. Monochromators and Optics

The required wavelength may be isolated by a filter, an interferometer or, more commonly, a dispersive monochromator (prism or diffraction grating). The materials used for the prism and windows depend on the wavelength range to be covered. A rock salt prism is usable over most of the region and KBr or CsI prism and optics for extension to 400 cm^{-1} (25 μm) or 260 cm^{-1} (38 μm), respectively. Alkali halide optics can be protected against the action of water by coating (up to 10 μm thick) with a synthetic plastic. Thin windows of Vitreosil ($\sim$5 cm diameter, 100 μm thick) (R11) and Mylar ($\sim$1.3 cm diameter, 25 μm thick) (S9) capable of withstanding high pressures and operating at low temperatures have been described. In the far infrared, almost the only suitable materials are organic polymers such as polyethylene and polyethylene terephthalate.

With most light sources short wavelength radiation is relatively more intense. To reduce stray light, most dispersive monochromators employ a double pass through a prism or use two diffraction gratings or a combination of a prism and a grating. A Littrow mount is frequently used in which a plain mirror behind the prism reflects the beam and returns it through the prism a second time. Greater dispersion can be obtained with a grating, but higher diffracted orders must be removed by filters,

a prism, or a second grating. A wide spectral range is obtained using interchangeable gratings. Transmission/reflection wire grating filters for use in the far infrared are being investigated (B4). Dispersive instruments are usually employed in the single channel scanning mode to generate a spectrum. Multicomponent analysis can be effected by fitting the monochromator with several adjacent entrance slits, spaced so that the radiation passing through the exit slit corresponds to the required absorption peaks. A rotating shutter opens each slit in turn and electronic circuits decode the modulated output signal.

Accuracy of wavelength calibration is maintained by thermostatic control of the monochromator. Water vapor may be harmful to optical components and in all instruments the internal atmosphere must be controlled by drying, gas filling, or evacuation. Carbon dioxide and water vapor absorb in the infrared and in single-beam instruments separate recordings of blank and sample spectra must be made. This is inconvenient, and double beam instruments, with automatic blank compensation and improved stability, are more commonly used.

Interferometric methods are particularly useful where high resolution and radiation throughput are required, as in the far infrared (S2). The signal they generate requires processing before a recognizable spectrum is obtained. A Hadamard transform spectrometer (see Section 3.4) has been described for use in the 1–2 μm range (D8). A simplified system could be used for the quantitative analysis of several components in a mixture where the absorption peaks are well defined. The advantage of the system is its greater information collection power, but it requires more elaborate data processing.

6.3. Sample Containers

Molar absorptivity in the visible and ultraviolet is often 10,000 or more, whereas in the infrared it rarely exceeds 1000. Consequently an infrared spectrum usually requires an undiluted or strong sample solution, or a long absorption path.

In gas analysis, multipass systems are used to give a long absorption path (P6). Vapor phase analysis avoids problems of solvent–solute interaction, but the absorption bands are subject to gas pressure effects. Some problems of gas analysis can be overcome by the matrix isolation technique, in which the gas mixture is diluted with nitrogen and deposited on a suitable window (CsI, CsBr, KBr, or NaCl) at 20K. Change of phase sometimes alters the spectrum, but with this technique the frequencies are said to differ only slightly from those in the gas phase, bands are significantly narrowed, and as little as 0.2 μmol of some gases may be detected (R10). The matrix must be inert, rigid, and

transparent, and solid nitrogen, argon, and xenon have been used. For the study of pure gases and vapors, cells operating down to 1K (S10) and up to 250°C (W22) have been developed. A windowless gas cell for operation up to 800°C uses inert gas "curtains" to contain the sample (S17). In clinical chemistry, gas phase analysis is potentially useful for the identification of drugs and other compounds but because of the complexity of infrared spectra the compound analyzed must be pure. Purification may be achieved by GLC but analysis of the effluent by infrared spectroscopy may be difficult (L13). It is usually necessary to collect a relatively large amount of the fraction and analyze its spectrum at leisure; reference spectra for this vapor phase may not be available. It is possible to interrupt the flow of carrier gas through the column until the spectrum of a fraction is recorded and then continue the elution.

For the analysis of liquids, cells are relatively simple and path lengths short. When solutions are measured, the reference cell can be used to provide automatic correction for solvent absorption. Increase in temperature reduces absorption at the band maximum and increases its width, thus for accurate measurement of spectra, temperature control is necessary.

Solids are usually ground with a material such as potassium bromide and compressed into a pellet. Moisture must be absent, and the transparent disk is placed in the window of the spectrometer. In general, however, measurement of intensity of absorption in the solid phase is unreliable due to scattering and reflection losses, and a uniform distribution of sample in the pellet is difficult to achieve. One method of handling solutions is to allow them to soak into a KBr wedge, evaporate the solvent, and compress the tip into a microdisk or pellet (C26); alternatively the sample (1 μl) may be either placed directly on the KBr disk (B13) or mixed with a little KBr powder and subsequently incorporated in the disk. These microdisk techniques can be used for the examination of gas chromatograph effluent. For multiple analyses, an enclosed turntable system loaded with the disks can be used.

6.4. Detectors and Data Processing

Thermal and photoconductive detectors are used to measure radiation intensities, but all have relatively slow responses and are subject to drift. The lead sulfide or telluride photoconductive cell has a response time of about 0.5 ms, but sensitivity decreases sharply above 2900 cm^{-1} for the sulfide and above 1700 cm^{-1} for the telluride. Thermal detectors are employed at longer wavelengths. The simplest of these is the thermocouple, which has a relatively slow response (about 60 ms), and several are usually linked to form a thermopile. Bolometers

and thermistors produce an electric signal as a result of a change in resistance of a conductor with temperature; their response time is about 4 ms. The Golay cell uses a pneumatic principle and is particularly suitable for the far infrared. The cell is filled with xenon and incident radiation causes this to expand and deform a diaphragm; external light is reflected from the diaphragm to a photocell which records movement of the diaphragm.

In nondispersive infrared gas analyzers, the detector is a cell containing a fixed concentration of the gas to be measured, diluted with an inert gas. Radiation from the source passes through the sample before falling on the detector. The energy absorbed in the detector at wavelengths corresponding to absorption bands causes heating of the gas and its rise in pressure is recorded. Alternatively the radiation absorbed in the detector can be measured by a thermal sensor. The amount of radiation reaching the detector is inversely proportional to the concentration of the gas in the sample. This principle can be applied to multicomponent analysis by using a number of detectors with different gas fillings. This type of detector is very sensitive to changes in composition of the sample which can modify the absorption of the gas to be measured.

The resolution of a scanning spectrometer depends on the recording speed, and the best quality spectrum is only achieved with slow scanning speeds and narrow slits. Since the energy of the source varies with wavelength, it is useful to be able to program the slit width, and sometimes the gain, for automatic scanning. Some instruments include an automatic suppression system which allows slow scanning rates when bands are being recorded, and faster scanning elsewhere.

Improved signal-to-noise ratios are obtained by stepwise spectrum scanning with signal integration at each wavelength (E4). More complex signal smoothing procedures using digital computation have also been used to achieve higher spectral resolution (P10). The most powerful tool for the fast production of high resolution infrared spectroscopy is Fourier transform spectroscopy (C18, L14). Digital signal processing has been used to produce derivative spectra. Correlation spectroscopy, wherein the incoming spectral signal is correlated with a replica spectrum stored in the computer, has been used for monitoring gas samples (D3).

6.5. Conclusions

Standardization in infrared spectroscopy is difficult (F10). As with all spectrophotometers, there may be nonlinearity of the detector and recorder and errors in cuvette calibration. Wavelength scales are usually

calibrated with substances such as water vapor (2000–1430 cm^{-1}), ammonia (1250–710 cm^{-1}) or methanol (830–420 cm^{-1}) with sharp easily recognized bands. Polystyrene film (3100–700 cm^{-1}) and indene (690–400 cm^{-1}) have also been used. A standard for infrared intensity measurements is more difficult to achieve, although a KBr pellet containing standard amounts of several substances can be used. At present it is doubtful whether intensity measurements are "absolute," and they cannot safely be applied to another spectrophotometer.

Digital computers are being increasingly applied to infrared problems (L17). They can be used to obtain accurate spectra by correcting for known instrumental distortions (C26) and for resolution of overlapping bands. The corrected spectra can be filed to provide an index of fine structure for both qualitative and quantitative analysis. Although infrared spectroscopy at present is primarily a tool for structural and qualitative analysis, the increasing availability of computer facilities for complex correction procedures may make more widespread quantitative analysis possible.

6.6. Raman Scattering

Raman spectra of molecules are generally simpler than their corresponding infrared spectra, and are generated by exposing the sample to monochromatic radiation, usually of optical wavelength. The scattered radiation contains lines at wavelengths different from that of the incident radiation. This change in wavelength arises from an exchange of energy between the molecule and the incident photon and corresponds to the energy of a molecular transition, the most important of which are vibrational processes. Raman spectroscopy may be used to determine molecular structure and for limited types of quantitative analysis (S15). The complete range of vibrational frequencies may be covered with one instrument. The linear relationship between the Raman line intensity and concentration makes quantitative analysis possible, but difficulties arise with colored or slightly fluorescent samples. In contrast to infrared spectroscopy, the solvent, in most cases water, has little effect on Raman spectra, though the tendency of most biopolymers to fluoresce is a limitation.

Since the intensity of Raman lines is about 0.01% of the source, spectra are generally weak and require spectrometers with high light-gathering powers. Apertures of about 4.5 are common, and larger ones have been used. In a typical arrangement, the sample tube, cooled by a water jacket, is irradiated with a bank of tubular mercury lamps which surround it. The scattered light is gathered from the end of the tube and passed to a prism or grating monochromator, the resolution of which

must be high (20,000) to detect wavelength changes of the order of 0.025 nm. Owing to the weakness of the signals, stray light must be eliminated by, for example, using a double monochromator or interferometer (P12). Photographic recording can be used, but photomultipliers are more common. Photon counting can be applied to measure low intensity signals, and, where fast spectra are generated, image intensifier phototubes coupled with storage video equipment have been used.

The greatest advance in Raman spectroscopy has been the development of the laser. These intense, monochromatic, polarized, small cross sectional area light beams are ideal sources (H15, H17). They have been used with special cells to observe spectra from −150°C to 800°C, and spectra have been obtained from nanoliter samples.

Although Raman spectroscopy may be used increasingly to elucidate structure of biological macromolecules (T4), its general use in clinical chemistry is not likely.

7. Micro- and Radiowave Spectroscopy

7.1. Electron Spin Resonance (ESR)

Atoms, ions, molecules, and molecular fragments with an odd number of electrons have characteristic magnetic properties. The unpaired electron has a spin and a magnetic moment, so that in a magnetic field it can take up two possible orientations which define two energy states. ESR (or electron paramagnetic resonance, EPR) is based on the splitting of these energy levels by the action of a magnetic field. The sample (solid, liquid, or gas) is placed in a strong stable magnetic field which can be slowly varied from 0 to 10^4 gauss and microwave energy (about 10,000 MHz) applied at right angles to the field. Unpaired electrons are excited and at the characteristic resonance frequency the energy absorbed from the rotating field causes them to "flip" from the lower to the higher energy level. The absorption of energy is detected as a reduction in the power of the transmitted radiation. The area under the absorption band is proportional to the number of unpaired electrons in the sample. Usually the first derivative of the absorption against magnetic field strength is plotted, as this gives a better discrimination between overlapping bands. Laser (W9), maser (C16), interferometric (A5), and cross-relaxation (W28) techniques have been used to increase the sensitivity of ESR. The instrumentation and some biochemical applications are described by Ingram (I1); it has also been used to study tissues (M3).

The technique has been used to study transition-metal complexes (M24) and reactions involving free radicals. Nitroxide radicals in solution tumble rapidly, giving characteristic sharp ESR signals. If the

radical is bound to a large molecule, such as an antibody, the spectrum is modified and appears as a broad envelope of peaks. This principle has been applied to "spin-label" drugs such as morphine for their determination by a "Free Radical Assay Technique" (FRAT) (L10). Antibodies are prepared in rabbits against a conjugate of morphine with bovine serum albumin. Morphine is spin-labeled by attaching a nitroxide radical and is then bound with the antibody. This reagent is added to the sample and any natural morphine present will displace the spin-labeled morphine, resulting in sharp ESR peaks. The intensities of these are a measure of the morphine concentration in the sample and the method will detect 1 ng of morphine in 25 μl of urine in less than 1 minute. Other spin-labeled compounds containing a nitroxide radical with a nitrogen atom bound to tertiary carbon atoms are described by Ingram (I1). Some of these could find an application in clinical chemistry.

The future use of ESR in clinical chemistry is difficult to predict, but in view of the biological importance of free radicals and the transition elements, further developments in this field may be expected.

7.2. Nuclear Magnetic Resonance (NMR)

Atomic nuclei with odd numbered atomic masses (e.g., ^{1}H, ^{13}C, ^{15}N) and with even-numbered mass but odd atomic number (e.g., ^{2}H, ^{10}B, ^{14}N) possess magnetic properties. They act like minute bar magnets, the axes of which coincide with the axis of spin. If the nucleus is exposed to radiation of appropriate frequency, transitions occur in which the nucleus "flips" from one orientation to another. NMR (or nuclear spin resonance, NSR) depends on the excitation of these nuclei in a magnetic field induced by radiofrequency (rf) radiation. The spectrum can be scanned either by changing the frequency of the rf oscillator or by varying the applied magnetic field; the latter is commonly used.

The NMR spectrometer consists of a nonmagnetic sample holder situated in the field of a strong electromagnet. Small modulation of the field is provided by sweep coils. Typically the field strength is about 14,000 gauss and must be homogeneous to 1 part in 10^8 within the sample area with comparable stability for short periods. Radiofrequency (100 kHz to 60 MHz) transmitter coils are located at right angles to the magnetic field, and a receiver coil, wound round the sample container, detects the absorption of energy. The magnetic field is gradually increased, and at characteristic field strengths energy is absorbed and the current flow in the coil increases.

NMR is primarily used for examining molecular structure and the proton is the most extensively studied nucleus (H12). The area under

the absorption band is proportional to the number of nuclei responsible for absorption in the sample, and the technique can therefore be used for quantitative analysis of a number of isotopes present in concentrations of 1% or less. It is rapid, nondestructive, and the ease of sample handling and greater certainty of identification make it particularly useful for problems which cannot be solved by other methods. Relatively simple, inexpensive wide-line instruments (B5) are available for use in quantitative elemental analysis and for studying the physical environment of nuclei. More complex high-resolution instruments are needed to resolve the fine structure of the absorption peak of a given nucleus. These use stronger magnetic fields provided by superconducting magnets (J4) and cooling of the resonant circuit to improve the signal-to-noise ratio (A2). Increased sensitivity is obtained with a short rf pulse and Fourier transform spectroscopy (F1). These improvements facilitate the measurement of ^{13}C used as a structural tracer isotope (R2). As NMR spectroscopy can be carried out over a wide range of temperatures and on liquids it is well suited to the study of biological compounds (B12, C9, W31), but its application to clinical chemistry is likely to be limited to special and as yet undefined problems.

8. Nucleonics and X-Ray Methods

8.1. Radiochemistry

The radiation resulting from the decay or transition of a radioactive nucleus may be α-, β-, or γ-rays. The nucleus may be identified by the nature and energy of its emission or the rate at which the radioactivity decays. As the output of the detector is frequently dependent upon the photon energy of the radiation, the need for a separate monochromator, with its associated energy loss, is avoided. Consequently, the efficiency of utilization of primary photons is high and only very small amounts of radioactive materials are required to generate a usable signal. For example, 10^{-7} Ci of ^{131}I (1 Curie (Ci) = 10^{10} disintegrations per second) corresponds to approximately 5×10^{-15} g of the isotope, and for this a simple analyzer system is adequate. Improvements in instrumentation are directed at greater stability, higher counting rates, and reduced background. If more than one radioactive element is present, measurement of the isotope of interest may be complicated by background signals arising from inadequate resolution of the analytical line and a continuum due to scattered radiation. Multichannel analyzers, in which the spectrum is divided into a number of energy bands, may be used to measure the spectral lines and the background radiation from a mixture. These instruments require greater resolution and are complex and expensive.

The instrumentation used in clinical chemistry consists basically of a detector, an energy analyzer, and a readout system (T9). It is frequently automated to handle large numbers of samples with counting times of a few minutes each.

8.1.1. *Detectors*

The three main types of detector depend on the use of photographic plates (autoradiography), the ionization of gases and the induction of scintillation in phosphors. Scintillation detection is one of the most convenient, as it is sensitive and the output pulse is proportional to the energy of the photon. The sodium iodide crystal activated by the introduction of about 1% thallium is the most widely used scintillator. The crystal may be shaped as a cylinder or a well. In liquid scintillation counting (G3, K18), the sample is mixed with the scintillator for measurement of α and β rays; modifications can be made to count γ rays (A12). Correction procedures have been devised to deal with any quenching (T1) and chemiluminescence (W29) which may occur. High energy β particles may be detected by their Cerenkov radiation, which arises when the particle travels through a transparent medium at a speed greater than that of light in the medium. This method avoids the chemical quenching of many scintillation methods (H8).

Ionization chambers, Geiger-Mueller tubes, and proportional counters all depend upon the electrical conductivity induced in a gas as a result of ionization (S15). Thin window or windowless gas flow proportional counters can be used for measuring ^{3}H and ^{14}C. If the sample can be converted into a gaseous form it may be incorporated into the counter gas to give almost 100% counting efficiency for ^{3}H. Semiconductors, such as crystals of silicon or germanium, can be used as detectors in a similar manner to the gas ionization chamber (S15). Where γ-ray spectrometry is used, as in activation analysis (see Section 8.2), the greater energy resolution of the lithium drifted germanium crystal is an advantage, although its low efficiency (about 10%) and small volume (about 100 ml) lead to reduced sensitivity (C19, C20). Iodine- and selenium-labeled compounds may be counted using a filter to separate the signals (M25).

Weak β emitters on paper and thin-layer chromatograms may be located by autoradiography (R3), whereas more energetic β emitters at higher levels of activity require scanning with a thin end window Geiger counter. Alternatively, the chromatogram can also be placed in a sandwich between two thin plastic scintillators (D17). The distribution of a γ- or energetic β-emitting isotope on a chromatogram can be determined using a wire spark chamber (N5).

8.1.2. *Electronics, Data Processing, and Automation*

The electronic system includes a power supply to drive the detector, a single or multichannel pulse height analyzer to select the pulses arising from the isotope to be measured and counting circuits to register the pulses. The main developments are toward smaller and cheaper units with greater stability, faster response (C15), and a wider range of facilities. Transistorized quench circuits have been used to improve the performance of the Geiger-Mueller counter (M9).

The data generated by counting systems is likely to need correction for background signals and counter efficiency, and the instrument may require calibration before the sample activity can be computed. These procedures are carried out either automatically within the instrument or externally, possibly with the aid of a computer (C7). A useful discussion of counting errors is given by Skoog and West (S15). The complex data produced from mixtures of isotopes measured by γ-ray spectrometry have been analyzed by a Fourier transform technique (I2).

Automation is essential for the analysis of large numbers of samples. Instruments can then be loaded with up to 400 samples and computer facilities used to give a print out of corrected activities. The sample chamber is usually temperature controlled and care taken to avoid large changes in background counts due to a wide range of sample activities.

8.1.3. *Applications*

In clinical chemistry the determination of stable elements by radiochemical methods offers no outstanding advantages over alternative methods, but the use of radioisotopes for determining organic compounds is developing rapidly. In isotope dilution methods (G6), a pure but radioactive form of the compound to be measured is mixed with the sample, a fraction is isolated, and its activity is determined. In radiometric or derivative analysis (W14), a radioactive reagent is allowed to react with the analyte; the labeled compound is separated and its activity is measured. The isotopes commonly used are ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{125}I, and ^{131}I. Radioimmunoassay combines the specificity of an immunochemical reaction with the sensitivity of isotope analysis (F4, S19) and is currently developing rapidly for the analysis of steroids, peptide hormones, and specific proteins (G9). Enzymes can be determined with labeled substrates (O1). The requirements of standardization and dosimetry make it probable that these methods will continue to be based on relatively large automatic instruments. However, the greatest problems are unlikely to be in the counting equipment but in sample handling and processing and in standardization of the system.

8.2. Activation Analysis

This technique is used to determine the concentration of an element by inducing radioactivity in one or more isotopes by nuclear particle bombardment. It can be used as a nondestructive method, but in a multicomponent sample, separation may be necessary to eliminate the effect of overlapping spectral lines. The method is similar in principle to other instrumental procedures that use energy sources to irradiate a substance to produce emission of characteristic radiation.

The activation source may be neutrons, charged particles, or γ-rays. Neutrons are most frequently used and are produced by nuclear reactors, isotope sources, linear accelerators, and cyclotrons. Some of these machines can also give intense sources of charged particles, such as protons, deuterons, and electrons, and other devices can provide sources of γ-rays. However relatively little use has so far been made of charged particles and γ-rays (E3) because the machines to produce them are not readily available. After bombardment, the γ-ray emission is measured by single or multichannel analyzers, either directly (B22) or after chemical or electrochemical (M5) separation to isolate the radioelement of interest. Test samples and standards are measured under identical conditions. When complex γ-ray spectra are generated, computer programs have been used to give simultaneous qualitative and quantitative analysis (T13).

Activation analysis is extremely sensitive and accurate, does not require a high degree of manipulative skill, and avoids many of the problems of contamination which often affect trace element analysis. At least 70 elements can be determined by this method, some in amounts as small as 10^{-13} g, and its application to biological fluids is described by Leddicotte (L5).

Since neutron generators and γ-spectrometers are expensive they are likely to be found only in large specialist centers, and hence the method is essentially a tool of limited interest to most clinical chemists.

8.3. Mossbauer Spectroscopy

The Mossbauer effect is the resonant absorption of low-energy γ-rays by nuclei bound in solids in such a way that there is no energy loss due to nuclear recoil. It depends upon the monoenergetic nature of the γ-ray emitted from an excited nucleus. When this ray falls on an unexcited nucleus of the same isotope, it will be absorbed if the nuclei are stationary relative to each other. However, if there is relative movement, there will be a Doppler shift in the frequency of the emitted γ-ray so

that it can no longer be absorbed. If the absorbing atom is in an environment which causes splitting of the nuclear energy levels (e.g., Fe in heme), and the source of γ-rays is accelerated and decelerated to produce emission of varying frequencies, some of these frequencies can be absorbed by the sample. The equipment consists of a radioactive source, which may be moved relative to the sample at constant acceleration, and a single-channel γ-ray detector to measure the intensity of the transmitted rays.

Many molecules in biological systems contain a transition metal such as iron which can be studied by γ-ray spectroscopy (D14, J3, L2). The Mossbauer spectra of two different forms of a molecule (e.g., oxidized and reduced) can yield useful information about electron transfer. The sample is usually in frozen aqueous solution. Additional information may be obtained by locating the sample in a strong magnetic field (3×10^4 gauss) which leads to splitting of the energy levels. The technique is complementary to single electron energy spectrometry for the investigation of molecular structure.

Although the Mossbauer spectrometer will not appear in the clinical laboratory for some time, if ever, it is conceivable that the very specific information it provides may become useful as diagnostic procedures become more refined.

8.4. X-Ray Spectroscopy

When electrons are ejected from the inner orbitals of an atom their replacement results in the emission of X-rays with energies characteristic of the electron transition. X-Rays are generated by electron, proton, α-, β-, γ-, or X-ray bombardment of a target or are emitted by radioisotopes. Their wavelength extends from 0.01 to 10 nm but the commonly used ones lie between 0.1 and 1 nm (12.4 and 1.24 keV). The spectrum of an X-ray tube is a continuum with superimposed lines corresponding to the elements in the target, whereas radiation from an X-ray emitting isotope is monochromatic. Specific X-radiation is usually selected dispersively, by an analyzing crystal or a grazing incidence diffraction grating, or with filters. X-Rays are detected and measured by Geiger, proportional, and scintillation counters and by photographic emulsions, phosphors, electron multipliers, and semiconductors. The amplitude of the output pulse of gas-flow proportional counters, scintillation and semiconductor detectors is a measure of the energy of the incident X-ray (F7).

X-Ray methods are used for qualitative and quantitative elemental analysis, and for determination of crystal and molecular structure, by measurement of the absorption, emission, fluorescence, and scattering of

X-rays. X-Ray diffraction techniques have been used to study crystallites in urinary calculi and bone and the structure of large organic molecules. X-Ray fluorescence analysis (B7, C1, C5) has detection limits in the nanogram range, but these depend upon the element determined and the sample matrix, which interferes with the analytical signal by modifying the intensity of the X-radiation from the element and by generating background radiation (J1). X-Ray fluorescence is a powerful, flexible technique for the determination of elements and, provided adequate correction procedures are made, is capable of good precision (CV $< 1.0\%$). The most important recent developments are radioisotope X-ray sources (C4) and semiconductor detectors (P4) with improved energy resolution. Lower detection limits should be achieved with more intense and stable X-ray tubes (J2). These developments could lead to simple nondispersive instruments (S25), suitable for the measurement of major elements in biological materials. Improved precision should result from automatic systems and computer data processing (V4).

X-Ray fluorescence is nondestructive and has significant advantages in simultaneous multielement analysis and ultramicroanalysis using electron beam excitation. It has found widespread industrial applications but as instrumentation is costly and complex in comparison with analytical atomic spectroscopy, the technique is not suitable for routine use in clinical chemistry. It seems unlikely that it can ever be more than a research tool.

8.5. Electron Probe Microanalysis

This instrument consists of a vacuum chamber containing an electro-optical system, which focuses a beam of electrons into a probe with a diameter of 0.1–5 μm at its point of contact with the surface of a solid sample. Electron excitation occurs and X-rays are emitted. The radiation is analyzed by combined wavelength and energy dispersion to identify the element, but quantitation (H14) is difficult due to problems in producing standards. By synchronizing the display of a multichannel counting system with the electron beam scan, the distribution of an element in the sample can be shown and, if necessary, related to morphology using an optical microscope. Some instruments are designed to be used for both electron probe microanalysis and electron microscopy.

The microanalyzer has been applied to biological materials (C1, D18), including the analysis of cell fractions (A6), measurement of titanium and zinc (10^6–10^8 atoms/cell) in leukocytes (C8), fluorine in teeth (W7), and calcium, phosphorus, and magnesium in small urinary calculi (C10). If electron probe microanalysis becomes a routine tool, it is unlikely to be located in clinical chemistry.

9. Particle Spectroscopy

9.1. Mass Spectrometers

Mass spectrometry involves the bombardment of molecules with a beam of medium energy electrons in a high vacuum and the analysis of the charged particles and fragments produced. The mass spectrum is a record of the relative amounts of the different ions present and is obtained by plotting the rate of ion collection against the mass-to-charge (m/e) ratio. Most commercial instruments analyze only positively charged particles although negative ion mass spectrometers are under investigation.

The gaseous sample, if necessary produced by heating, is allowed to leak into a low pressure chamber (about 10^{-5} Torr), where it is ionized. Any carrier gas must be removed by filtration. Special sample handling devices include leak inlets for gases (H9), ampoule and oven systems for liquids and solids (P1), and sparks for vaporizing solids and frozen aqueous solutions (O2). The ion source is usually a tungsten filament from which electrons are drawn through positively charged slits, accelerated by an electrical field and directed at the passing gas stream, where they produce ionization and fragmentation. The positive ions are accelerated by an electrostatic field and passed through slits which resolve them according to their m/e ratios. Ions are focused on the exit slit by varying their velocity, via the accelerating potential, or the magnetic field. Various types of ion separator systems are used, with either single focusing using a magnetic field or double focusing with an additional electrostatic field. Time-of-flight separators do not use magnetic separation but a field-free drift tube, 1 m long, arranged so that the lighter particles arrive at the detector before heavy ones. In the quadrupole spectrometer, four short parallel metal rods are arranged symmetrically round the beam. One pair is connected to a positive dc source and the other to the negative terminal, and an additional rf ac potential is applied to both pairs. Positively charged particles are not accelerated but oscillate about their axis of travel, and only those with certain m/e ratios pass through to the detector. Ions are usually detected by the current they generate in an electrometer. The photographic plate is useful for a wide mass range of ions, but the response of the plate depends on the ion striking it (V5). Electron multipliers are highly sensitive detectors and can be used to obtain lowest detection limits (Y1). A spectrum can be obtained from about 1 μmol of sample, and sometimes less, with detection limits down to 10^{-11} g for some compounds.

Mass spectra are usually simpler than emission, absorption, or fluores-

cence spectra, and the technique can be used for structural and qualitative work as well as quantitative analysis (W13, W19). The peak of the highest mass number detected corresponds to the parent molecule minus one electron, and this provides an accurate method of measuring molecular weights. The family of particles with a range of mass distributions is often characteristic of the parent compound. Recent developments in instrumentation (D11, R6) have resulted in a range of commercial mass spectrometers, the cost of which is related to their resolution and mass range. Expensive high resolution instruments with double focusing are required for structural analysis, but for quantitative analysis cheaper low resolution instruments with a limited mass range are satisfactory. Time-of-flight and quadrupole spectrometers are smaller, more mobile, and less expensive than those which use magnetic focusing, but their resolution, reproducibility, and ease of mass identification are less satisfactory. Computers have been used to identify compounds from their mass spectra (B6). The combination of gas chromatograph–mass spectrometer and computer is an extremely powerful tool but at present prohibitively expensive for widespread use (H23). The speed of analysis might be increased by using a mass spectrometer incorporating Fourier or Hadamard transform techniques, linked to several gas chromatographs. Such an instrument could become economically viable in a large clinical chemistry laboratory in future.

9.2. Electron Spectroscopy

In this technique an electron is ejected from a sample by a photon or electron of known and relatively low energy. The emitted or scattered electrons are sorted by a magnetic or electrostatic analyzer according to their energies and then detected by an electron multiplier. Electron spectroscopy can be used to measure the elemental content of surfaces and films to a depth of about 5 nm or to study the structure of complex molecules in the gas or solid state. Surprisingly its application to biological problems has been neglected except for an investigation of protein structure (K17).

Techniques that fall within the general heading of electron spectroscopy for chemical analysis (ESCA) include photoelectron spectroscopy of inner shell electrons (PESIS), photoelectron spectroscopy of outer shell electron (PESOS), and Auger electron spectroscopy (C2, H16, P11). The advantages of ESCA are high sensitivity, easily interpreted spectra, and that the sample is not destroyed (H27). A few commercial instruments are now available.

10. Chromatography

Chromatography is a technique for separating mixtures of chemical compounds prior to their identification and determination. It depends upon the distribution of a solute between moving and stationary phases which are in contact. The two main types are gas–liquid chromatography (GLC), which uses a mobile gas phase and a liquid adsorbed onto a solid as stationary phase, and the various forms of liquid chromatography, which include partition, adsorption, ion exchange, and gel permeation. All types of column chromatography have shown major developments in recent years (J6, S21, W3, Z1) and the simple homemade column, supplemented by accessory fraction collectors and monitors, is rapidly being replaced by commercial systems that will undertake the complete analysis. As both gas and liquid chromatographs have many common features, their major components can be considered together. Paper and thin-layer chromatography (TLC) are open-bed variants of liquid chromatography and use a thin layer of supporting medium instead of a column. They require different instrumentation and will be dealt with separately.

10.1. Column Chromatography

10.1.1. *Sample Pretreatment*

With many sensitive liquid chromatographs, little or no pretreatment is necessary, but with GLC partial purification and isolation are required. Since most biological substances have low volatility or poor thermal stability, it is usually necessary to prepare suitable volatile and stable derivatives before analysis. At present, pretreatment methods involve little or no specialized instrumentation, but this will undoubtedly be developed for use with faster automated chromatographs. Methods of sample injection for GLC are relatively crude and an internal standard is necessary to compensate for errors in the volume injected. If large numbers of samples are to be analyzed repeatedly, some form of automatic injection, linked to a timing device, will need to be developed (J6, Z1).

10.1.2. *Columns*

The efficiency of separation in all types of column chromatography depends on the extent to which the sample bandwidth broadens during development of the chromatogram. Three factors determine this broadening: (1) axial diffusion of solutes within the mobile phase, which is

reduced by fast flow rates, thus allowing less time for diffusion; (2) transfer of solutes between the phases, which is minimized by slower flow rates; (3) inequalities in the length of the flow path of the mobile phase, which depends on the particle diameter and packing of the supporting material. For liquid chromatography (G4), the highest resolution and speed will be obtained with small and regular sized particles and long narrow columns, and these necessitate forced flow of the mobile phase either with pumps, which tend to give a pulsed flow, or with compressed gases, such as nitrogen.

The two basic types of column used in GLC are the packed column, in which the supporting material is previously coated or impregnated with liquid phase, and the open hole tubular column, consisting of a long fine tube of which the inner surface is coated with a uniform layer of stationary liquid phase. Packed capillary columns (K3) are manufactured by drawing out a packed glass column into a capillary and then forcing through a solution of stationary phase. Micro-packed columns (C22), with diameters down to 0.6 mm, are wound to a helix before packing with a supporting material impregnated with the stationary phase. The theoretical performance of these types has been compared (C22). It seems probable that capillary columns will replace packed columns because they are capable of higher resolution and speed of analysis, but they are difficult to prepare and probably best purchased ready-made. At present, column preparation is the most important factor in achieving reliable and reproducible analyses (S21). Capillary columns are particularly useful with systems using the mass spectrometer (GLC-MS), as the low bleeding of the stationary phase gives only negligible background to the spectrum. The range of solid supports for GLC (P2) includes diatomaceous materials, fluorocarbons, and specialist materials, such as glass beads, vermiculite, and porous polymer beads. By careful selection, the range of columns used in routine practice could probably be reduced to a few of wide application.

High-performance liquid chromatography has developed from GLC and employs many of its features. These include high resolution, speed, ease and simplicity, continuous monitoring of the effluent by a range of detectors, identification based on retention time, repetitive analyses on the same column, and automation of both the analysis and calculations. High resolution automated liquid systems may use pressures up to 5000 psi in stainless steel columns 150 cm to 1 m long and with internal diameters of 0.15 to 0.22 cm (B24, F5, K6, P8). The tube may be folded or coiled in a helix and electrically heated to 60°C. Only 5 g of ion exchange resin, of 12.5–13.5 μm diameter, may be needed. A volume of 50–200 μl of body fluid is injected through a valve, and gradient elution used with constant

displacement pumps capable of delivering 200 ml per hour. Parameters such as pH, temperature, and flow rate can be adjusted to give optimal separation.

Several new types of supporting material have been developed for liquid chromatography, some of which are also applicable to GLC (K13, K14, P5). The stationary phase may be chemically bonded to the support thus counteracting the leaching effect which occurs when the mobile phase flows past a stationary phase impregnated in but not strongly held by the inert support. Another type uses an impervious spherical particle to which a thin layer of porous coating is attached, so that the chromatographic separation occurs only on these surfaces and diffusion of solutes within the stationary phase is reduced. In high pressure liquid systems, pellicular ion exchangers (H24, W3), prepared by coating glass beads with an ion exchange resin, can separate mixtures with a speed and resolution similar to those obtained with GLC. Packings with chemically bonded stationary phases will probably become increasingly important in the future. It is likely that a selection of relatively few packings will enable a wide variety of chemical species to be separated, the selectivity of separation being controlled by altering the mobile phase (K14). Provided that regular packing occurs, a decrease in particle size of 50% will double the efficiency of the column if its length is kept constant, with a 4-fold increase in pressure (H13). Methods are available for giving regular beds with particle sizes of 50 μm.

Some highly efficient liquid systems use a heated column; a 1°C rise in temperature can alter the retention time by 2–3%. The viscosity of liquids decreases as temperature rises, so solvents are easier to pump and faster flow rates are possible. However, precise temperature control, preferably to ± 0.2°C (K14), is necessary for reproducible performance and to secure baseline stability by maintaining a constant solubility between the stationary and mobile phases. With GLC, it is possible to alter the retention time 1000-fold by increasing the temperature. The analyst is then faced with the conflicting requirements of temperature stability, to ensure a reproducible retention time, and the ability to vary it rapidly according to a predetermined program in order to elute the material it is required to analyze.

Various types of column can be combined. Sequential analysis with liquid chromatography can first pass the sample through a gel to separate constituents according to their molecular size, followed by some form of affinity chromatography using partition or adsorption. A two-column GLC system for steroid analysis has been described (H22), in which the sample is first introduced to a high-capacity low-resolution column for a preliminary separation. After a suitable time, the flow of gas is re-

versed, the temperature rapidly increased and the components passed to an analytical column with a high resolution but low capacity. Linked columns of this type, under automatic control, should be capable of handling many more samples and will also remove excess reagents and solvents which might otherwise damage sensitive detectors. Another method of avoiding this solvent front is to incorporate a bypass in the gas stream for removing solvents and reagents, or alternatively introduce the sample on a metal or glass carrier (H22).

10.1.3. *Detectors*

Most of the carrier gases used in GLC are inert, so a wide range of detectors can be used (G7, H6, J6). The majority of these are nonspecific and the principal requirements are for high sensitivity, a quantitative response, high signal-to-noise ratio, and freedom from drift. The two main types are the thermal conductivity detector (or katharometer) and the various forms of ionization detector. The thermal conductivity cell contains filaments forming a Wheatstone bridge that measures the difference in thermal conductivity between the stream of carrier gas containing the sample components and a reference stream of carrier gas before the injection point. Ionization detectors depend on the conduction of electricity by ionized particles produced by burning the carrier gas, by ionizing radiation, by electrical discharge or some other means. In the flame ionization detector, hydrogen is mixed with the carrier gas and burnt. The tip of the burner jet functions as a collector electrode. If argon is used as a carrier gas, it can be bombarded with β particles from a strontium-90 source, which raises the argon molecules to a metastable electronic level. When these collide with sample molecules, energy is transferred and ionization occurs. In the electron capture detector, β particles from a tritium source are used to produce ionization of the nitrogen carrier gas and formation of "slow" electrons; when collected, these produce a steady baseline current. The introduction of an electron-capturing gas or vapor into the sample stream causes a decrease in current, which is a measure of the amount and electron affinity of the components in the carrier gas.

Methods have been described for GLC (S21) in which the effluent stream is split and monitored by different detectors. This could be useful in providing two monitors, of different sensitivities, if a wide range of signal intensities were expected. Combinations of nonspecific detectors with specific ones (e.g., for detecting isotopes used as tracers) do not yet seem to have been tried, but it is likely that these will be developed in the future. Most detectors require a specific calibration factor for each substance, usually in terms of some suitable internal standard. Martin

(M6) has suggested that this is unnecessary and that it should be possible to destroy at least part of the sample and then carry out some form of elemental analysis for at least carbon and hydrogen and possibly for nitrogen, oxygen, and other elements.

The mass spectrometer is the most powerful GLC detector used so far. However, the GLC-MS combination is expensive for routine and repetitive analyses but is invaluable for rapid identification of unknown peaks. It has been widely applied in steroid analysis (B16, H23), identification of drugs (L4) and for the identification and quantitation of unusual metabolites in some inborn errors of metabolism (C23). The high sensitivity and specificity of the GLC-MS combination enables all metabolites in a urine to be unequivocally identified. Inexpensive mass spectrometers, with a limited range of m/e values, are becoming available and could become useful for repeated analyses of similar samples for a limited range of constituents.

In liquid chromatography there are four main classes of detector (C17):

1. Differential refractometers monitor the difference in refractive index between the pure mobile phase and the eluted fractions. They are simple, sensitive and do not destroy the sample, but temperature control is critical and, since they depend on the constancy of the mobile phase, they are difficult to use with gradient elution.

2. The heat of adsorption detector monitors the rise in temperature which occurs when eluted solutes are adsorbed exothermically. A constant flow rate is essential, together with efficient thermostatting, and the endothermic adsorption of one solute may interfere with the detection of the exothermic adsorption of the next.

3. Transport-ionization detectors use the flame ionization detector common in gas chromatographs. Although expensive, these have a high sensitivity, quantitative response over a wide range and are not influenced by temperature or flow rate of the mobile phase. They can be used with gradient elution. The moving wire detector (S4) collects a thin film of column eluate on the surface of a moving wire. After evaporation of the solvent, the solutes are burnt in oxygen. The carbon dioxide formed is mixed with hydrogen and passed over a nickel catalyst, and the methane thus produced is detected by a flame ionization detector. This method is claimed to give a linear response over 4 orders of concentration range and to detect approximately 1 μg of organic solute per milliliter of mobile phase.

4. Photometric detectors are widely used; recent versions (T2) require only small volumes (less than 10 μl) of eluate in a flow cell, and the sample may be recovered. A spectrophotometer can be used to produce

a spectrum for identification purposes, or alternatively the eluate may be monitored at one or more wavelengths using, for example, a simple ultraviolet colorimeter. The high sensitivity and low dead volume enable peaks of only nanogram amounts of materials to be detected (K12). This type of detector can be more specific, but the solvent must be transparent at the wavelength used. Ultraviolet detectors are inherently more sensitive than refractometers, but the two together can provide more information than either separately, and on occasions may detect two peaks which are incompletely resolved by the chromatogram.

10.1.4. *Data Handling*

The detector output is usually monitored with a recorder, and it is then necessary to measure the retention time for identification purposes and to integrate the peak area (which is a more sensitive index than peak height) to calculate concentration. An amino acid analyzer has been described (M18) in which peak height can be made directly proportional to concentration. This simplifies calculations and is claimed to give better precision than measurement of peak area. The accuracy and precision of all types of column chromatography depends on the control of temperature and flow-rate as well as the shape of the peak, noise, and drift in the detector. A computer can be used to resolve overlapping peaks and compensate for the inadequacies of the chromatographic separation, but for routine work this is expensive. A computer with a GLC-MS system enables mass spectra to be recorded and stored and calculations made of individual ion abundances; from these the parent molecules can be identified and measured (B16). With liquid chromatography, on-line computers have been used to overcome the problems of detecting, resolving, and measuring areas of overlapping peaks (C11). One capillary column system, using 12 columns with gradient elution, linked the colorimeters on-line to a computer (V3).

10.1.5. *Conclusions*

One of the main advantages of liquid chromatography is the ease with which the eluate can be monitored, either in series or in parallel, by different detectors, including photometers at several wavelengths, fluorimeters, and radioactive counters. With GLC, most detectors are nonspecific and are influenced by extraneous materials in the eluate. Liquid chromatography can be used with nonvolatile and thermolabile substances, whereas GLC requires the preparation of a volatile and heat-stable derivative. Only about 20% of known compounds lend themselves to GLC (K14). Liquid columns appear capable of handling higher loads, are relatively easy to automate, and are being widely used for analysis of

complex biological mixtures. One version can complete a run for organic acids in 0.2 ml serum in less than 6 hours (R13), and another will analyze picomole quantities of nucleic acid components (B24). Automated commercial systems for amino acid analysis have been available for some time (J5), and most of these employ programs for automatic sample loading, control of gradient elution and regeneration of the ion exchange resin column. Additional types will undoubtedly be developed for other applications, but since each group of compounds is likely to require different conditions for their separation and measurement, such instruments will be dedicated for specific tasks and not readily adaptable for other purposes. New fast liquid systems are being developed for coupling to a mass spectrometer (R13), and these would have the speed and capabilities similar to the most advanced GLC-MS systems, but with better resolving power and the ability to be used with thermolabile substances.

The outstanding feature of GLC is its high sensitivity in relation to other techniques. In addition, the use of a mass spectrometer as a detector provides a unique facility for identification of unknown compounds. As with liquid systems, current instruments are mainly used for the analysis of a limited number of related compounds in a single sample giving, for example, a metabolic profile for steroids or other compounds (H23) which would be difficult to obtain by other methods. There is a need for further high capacity high resolution automated GLC systems capable of analyzing large numbers of samples routinely. These will need to incorporate some form of back-flushing to return the instrument to a standby condition, together with simple methods for removing any nonvolatile matter introduced with the sample (M6). Another requirement not currently met is a simple method of selecting a peak and rerunning it on a different stationary phase in order to further separate its constituents and study their chemical nature (M6). The clinical value of analyses made with column chromatography will no doubt act as a stimulus to the development of improved instruments for the future.

10.2. Paper and Thin-Layer Chromatography

The potential clinical value of the analysis of complex mixtures such as amino acids and steroids is so great that any method must be capable of dealing with large numbers. There is a limit to the speed at which any type of column chromatogram can be run, and the fastest column systems are usually dedicated to one task, as well as being complex and expensive. In contrast, the most attractive features of paper and thin-layer chromatography are their ease and simplicity, requiring little or no instrumentation, and allowing the do-it-yourself enthusiast endless oppor-

tunities to display his skill. The technique is fast and can deal with large numbers of samples and virtually the only disadvantage is the difficulty of obtaining good quantitation.

A wide range of papers and media have been described (W3, Z1), including modified celluloses with ion-exchange properties, resin impregnated papers, and polythene backed paper which often improves the spots developed. The inherent variation between different pieces of paper necessitates the use of standards on each sheet for best results. A number of devices are available for automatic and multisample application, and these can improve both speed and accuracy (Z1). To achieve fast and selective separation, other accessories have been used, including solvent gradients, vapor programming and automatic methods of starting and finishing the run, but these have all been at the expense of the inherent simplicity of the method (Z1).

There are four basic methods for determining the amount of substance present in a spot (L6): (1) analysis of the eluted spot, (2) direct densitometry by reflected or transmitted light, (3) measurement of ultraviolet-excited fluorescence, and (4) measurement of fluorescence quenching. Sensitivity can sometimes be improved by labeling the substance by a reaction which introduces an easily detectable grouping, such as a radioactive element. Fluorescence reactions with steroids have increased the sensitivity of the method so that it compares favorably with that of flame ionization in GLC (B27). A variety of instruments are available for use with these methods (L6, Z1). Bush (B25, B26) discussed the general principles of automation of steroid analysis and concluded that direct photometric scanning of paper chromatograms was the most productive method and superior to any other form of chromatography from the logistic viewpoint. He suggested that the poor accuracy and precision of the final measurement were due to two factors: (1) the optical pathway was seldom appropriate for quantitative absorption or fluorimetry; (2) reagents were frequently too dilute to achieve a stoichiometric reaction over the desired range of concentration. Bush described an automatic scanner, linked to a computer, which was capable of processing 500–600 45 × 5 cm paper chromatograms in a working day. Other automatic scanners have been described which are applicable to either light-absorbant or fluorescent substances. Boulton (B10) concluded that two types of scanner were required—a sturdy, cheap, and fast filter instrument which could be used for absorbance or fluorescence on a variety of media, and a more advanced instrument with a monochromator for dual wavelength scanning and electronic devices for smoothing and subtracting the signals. There is considerable potential for applying laser beams, polarized light and fiber optics to future instruments of this type (B10).

11. Electrophoresis

Electrophoresis depends on the movement of charged particles in an electric field. Many different media have been used (S22), including cellulose acetate, paper and gels of starch, agar, silica, Sephadex, agarose, and polyacrylamide, arranged in sheets, slabs, disks, or thin layers. Different solutions and techniques have been used to provide density gradients (L16), pH gradients, and gel concentration gradients (W30), all of which can give a more refined separation of components. The initial separation in one direction may be followed by chromatography, immunodiffusion, or further electrophoresis in another direction.

The equipment for each of these variants is basically similar. Reproducible separation of molecules requires a smooth and constant voltage gradient within the supporting medium. With most constant voltage power packs, the proportion of voltage drop available for electrophoresis varies with changes in resistance in the medium and changes in the external circuit. A sensing and control system has been described (D16) which can be used with different power packs, over a wide range of voltages, to produce a constant potential over the separation field. Electrophoresis with gels may use relatively high voltages, and some instruments use a cooling liquid circulated through a gasket or between plates. Others separate the electrodes from the medium by membranes which ensure that evolved gases do not disturb the medium. High voltage electrophoresis may use voltages of at least 4000 V, and cooling and safety then present special difficulties.

The outstanding problems of electrophoresis are in identifying and quantifying the separated components. Gels are difficult to handle, and many workers merely photograph the stained gel *in situ* after electrophoresis. Alternatively the stained gel is cut into segments for assay of the fractions. Ultraviolet photometers have been used to detect proteins separated by isoelectric focusing and electrophoresis. An AutoAnalyzer method for density gradient electrophoresis has been described (L16) in which serum is diluted with a sucrose-containing buffer and injected into an electrophoresis chamber containing cellophane membranes to separate the electrode compartments. Electrophoresis is carried out for 17 minutes, and the separated proteins are detected by scanning with a photometer at 280 nm.

Many commercial instruments are available for scanning stained electrophoresis strips. One of these (V2) operates at high speed and includes automatic zero adjustment, digital readout of the protein fractions and displays the electrophoretic pattern as a tracing on the fluorescent screen of a cathode ray tube.

It seems probable that in future, electrophoresis of plasma proteins on paper or cellulose acetate strips will only be used as a screening technique for detecting gross abnormalities. Improved instrumentation will be needed to process large numbers of samples automatically. Analysis of urinary proteins would be facilitated by the development of improved methods of concentrating the proteins prior to analysis. Specific immunochemical methods provide more information about individual proteins, but instruments for quantitation of immunodiffusion and immunoelectrophoresis are largely lacking. Continuous-flow methods for specific protein determination have been described (K8, R9), in which the light scattering produced by antigen–antibody complexes is measured. Instrumental methods of this type, which enable large numbers of analyses to be performed automatically, will become more important for protein analysis in the future.

12. Electrometric Methods

Many electrometric methods of analysis are described in the literature (W18), but this section will be limited to the few which have been widely used in clinical chemistry.

12.1. Membrane Electrodes

Ion selective membranes measure ion activity (a) which is related to concentration (c) by

$$a = \gamma c$$

where γ is the activity coefficient. The nature of the sensing membrane determines the selectivity of the measurement and the concentration range. In the ideal situation, the membrane allows only the ion of interest to pass from the sample solution at the outer membrane surface to an internal solution in contact with the inner membrane surface. An electrical potential develops which can be measured by making electrical contact to the inner solution with a suitable reference electrode, and connecting the sample solution with a second reference electrode via a salt bridge. The voltmeter indicates the potential (E) according to the (simplified) Nernst equation:

$$E = \text{constant} + S \log a \tag{3}$$

where S is a temperature-dependent constant. In practice, membrane electrodes differ in their selectivity and their usefulness depends on the other ions present in the sample. If A is the activity of the ion it is required to measure, and B the activity of a second interfering ion, Eq. (3) is modified to (R14):

$$E = \text{constant} + S \log (A + k_{AB}B) \quad (4)$$

k_{AB} is the selectivity ratio (or constant) and expresses the relative response of the electrode to ions A and B.

As the potential developed at a membrane varies logarithmically with ion activity, some instruments use logarithmic pA scales, where pA = $-\log(A)$, or alternatively use antilog circuits to enable activity to be read directly from the electrode potential reading. These direct reading instruments are rapid and require little or no pretreatment of the sample, but their main disadvantages are drift and the difficulties of selecting suitable standards for calibration (B2, R14). Some ion-specific electrodes can be used as end point detectors in potentiometric titrations. This avoids many of the difficulties of calibration and is usually more accurate, since the change in potential rather than its absolute value is measured.

Any phase boundary which responds to an ion concentration in a reproducible manner according to the Nernst equation can be used for the electrometric determination of that ion (S18). A wide variety have been described (B3, P13, R14, S18), but only those which have been successfully applied to biological systems will be considered here.

12.1.1. *Glass Electrodes*

These respond to monovalent cations and are most sensitive to H^+ and Ag^+. The composition of the glass determines the selectivity of the electrode, e.g. (D1):

pH—22% Na_2O, 6% CaO, and 72% SiO_2
Na—11% Na_2O, 18% Al_2O_3, and 71% SiO_2
K—27% Na_2O, 4% Al_2O_3 and 69% SiO_2

None have yet been found to respond to anions or divalent cations.

Many different types of glass electrode and cell assembly are in use for blood pH measurements, and some give results differing by as much as 0.1 pH unit on the same sample (K5). The main sources of error arise from temperature variations, the technique of standardizing and washing the electrode, and variations in the liquid junction potential, which depends on the structure of the junction and the composition of the individual blood samples (B3, K5). A strong KCl solution is usually employed as a bridge between the blood sample and the calomel reference electrode. For maximum stability of the liquid junction potential, the KCl concentration should be high, but this inevitably contaminates the sample and causes protein precipitation at the boundary. Replacement of the KCl bridge by one of isotonic saline would reduce these effects, but this results in a variable shift in apparent blood pH, and there is at present

no method of distinguishing between the two values (K5). Although pH meters will detect a change of 0.001 unit, and blood pH measurements have a repeatability of ±0.01 unit, the uncertainty of measurements is believed to be ±0.03 to ±0.05 unit, probably as a result of a residual liquid junction potential (K5). A method has been described (M1) in which corrections are made for the systematic differences between the results obtained with a microglass electrode and a hydrogen electrode, and this is claimed to have a reproducibility of 0.002 unit.

Highly specific sodium electrodes have been developed in which the selectivity for sodium may be 10^5 times greater than that for potassium (C3, M19). With urine, the pH and potassium concentration should preferably be controlled, but this is unnecessary for blood. The potassium glass electrode is less selective and responds to NH_4^+ and Na^+. Its selectivity may vary with age (M19). It can be used with blood only if corrections are made for sodium concentration according to Eq. (2) (M19, N2, N3), but when this is done, the electrode shows a linear response to potassium concentration. The precision of serum sodium and potassium measurements with electrodes was found to be better than those obtained by flame photometry (M19, N3). To compare the accuracy of the two methods, the results by flame photometry must be converted to concentrations in serum water. For most specimens, it was found that concentrations could be calculated satisfactorily from activity measurements and results by the two methods agreed (N3), but differences were noted with some samples. So far the cause of this has not been resolved, but it is possible that in future ionic activity will be recognized as a better diagnostic feature than ionic concentration (N3).

An electrode for measuring urea has been described (G11), consisting of a thin film of urease, immobilized in acrylamide gel, on the surface of a glass electrode responsive to NH_4^+. Conditions are carefully selected to ensure stability of the enzyme, and the potential developed is proportional to the logarithm of the urea concentration. Blood glucose and lactate have been determined with a membrane electrode in which the enzyme (glucose oxidase or lactate dehydrogenase) is trapped in a porous or jellied layer at the membrane surface (W20).

12.1.2. *Ion Exchange Membranes*

Many porous ion exchange membranes with high cation or anion selectivity have been described (B3, P13, S18). Sparingly soluble crystalline materials have been used as anion sensors, the membrane consisting of single crystals or pressed pellets often embedded in a vulcanized silicone rubber matrix. Examples include electrodes for fluoride (LaF), sulfide (silver–silver sulfide), iodide, and sulfate. These probably function as

ion exchange membranes, and in biological fluids all suffer from protein poisoning. Clay membranes have been used for Ca and Mg, and a number of others are based on ion exchange resins. Permselective collodion matrix membranes of high cation and anion selectivity have also been described (S18). Several examples of organic anion sensitive electrodes have been developed (e.g., for salicylate), but so far these lack specificity (H10).

In liquid ion exchange membranes the sensing membrane is the interface between an organic fluid and the sample (S18). An inert supporting material such as a permeable film or ceramic plug maintains the integrity of the interface. The nature of the organic liquid determines the ions sensed and the concentration range covered. These liquids are solutions in water-immiscible solvents of substances with an inorganic group attached to a suitable organic molecule, usually with a molecular weight of 300–600. Typical ion exchange compounds are the secondary amine, *N*-lauryl (trialkylmethyl) amine, and acidic compounds such as monodioctylphenylphosphoric acid. Calcium and potassium electrodes have been developed with capabilities not offered by glass. One calcium electrode, using the calcium salt of dodecylphosphoric acid, has a selectivity for calcium 1000 times that for sodium and potassium and 100 times that for magnesium, and is finding wide application for determination of serum ionic calcium (L11, M20). Preliminary trials have been made of liquid ion exchange electrodes for potassium (N2, W26); a liquid sensor using valinomycin in an aromatic solvent has a selectivity for potassium 5000 times that for sodium (P7) and shows considerable promise for use with biological fluids. Two types of chloride electrode are available, one using a liquid ion exchanger and the other a solid state silver chloride membrane (B2); at present, the latter type is employed in commercial chloride meters used for sweat analysis (H4). Liquid electrodes have been described which are selective for individual amino acids (M8).

Other new sensory membranes are being developed for use with neutral molecules, but despite the time and effort spent on developing specific ion exchange membranes, they have so far found only limited application in clinical chemistry. Liquid ion exchangers are more promising and as well as being extremely versatile are said to be easier to manipulate. Their future role will depend on improvements in their selectivity (R14).

12.1.3. *Carbon Dioxide and Oxygen Electrodes*

These both depend upon the use of membranes permeable to the gas but not to ions. The Severinghaus CO_2 electrode (S5) uses a glass elec-

trode to measure the pH of a film of $NaHCO_3$ solution separated from the sample by a Teflon or silastic membrane. The system is calibrated directly by passing gases of known pCO_2 into the cuvette. The main sources of error are membrane leaks, the introduction of air bubbles from the wash fluid, and sampling and temperature errors.

Various types of oxygen electrode have been described (L8), but the most widely used is the Clark polarographic electrode, consisting of a platinum cathode and a silver/silver chloride anode. The platinum wire (usually 10–25 μm diameter) and silver wire are sealed in glass so that only their cross sections are exposed. When a potential of 0.6–0.7 V is applied, oxygen is reduced at the negatively polarized surface, and the current through the cell is a linear function of the oxygen tension in the solution bathing the electrodes. Proteins can poison the Pt surface, so the test solution is separated from the Pt-electrolyte cell by a polyethylene or polypropylene membrane through which oxygen can diffuse. The current produced by the reduction of oxygen is dependent on the pH of the electrolyte, and since CO_2 can also diffuse through the membrane, the electrolyte is usually buffered. The use of a very small cathode reduces oxygen consumption and thus the formation of oxygen gradients in the solution, so that stirring is unnecessary. It also gives a faster response and greater linearity but the smaller current requires more amplification for measurement (G1, S5). Temperature control is critical, and a 1°C change results in an error of 10% (S5). The calibration and use of this electrode is described in detail by Gambino (G1). The silver electrode is not readily sealed into glass, and this imposes limitations on the configuration of the electrode which can be important in studying tissue metabolism. For this, Clark and Sachs (C13) have described a micro electrode with two very fine Pt wires sealed in glass and separated by a capillary thin layer of iodide-containing electrolyte. The applied voltage is slowly alternated so that each electrode is in turn the cathode.

The polarographic oxygen electrode has been used to measure the rate of oxygen consumption during the reaction of glucose with glucose oxidase (K1, M2), catalase being added to break down the hydrogen peroxide formed. The sensor gives a signal proportional to the oxygen activity in solution. The method is fast, sensitive and although it does not require protein precipitation, it is best used with plasma rather than whole blood. The same principle can be used to measure other substances, such as uric acid, for which there is a suitable oxidoreductase enzyme (M2).

12.1.4. *Automated Systems*

Electrode systems for the rapid measurement of blood pH, gases, and ions would be of great value in intensive care units where these analyses

are required frequently during the day and night. One "self-service system" (S5), designed for use by nontechnical personnel without special training, measures pH, pCO_2, and pO_2 with push-button valves to control the flow of calibration gases, wash fluids, pH buffer, and saturated KCl. A more complex system incorporated Na and K electrodes and was linked to a computer (N3) and results could be obtained 3.5 minutes after injection of 2 ml of blood. Although automated commercial systems for pH, pCO_2, and pO_2 are available, there are difficulties in selecting suitably stable Na and K membranes, together with the problems of calibration.

12.2. Polarographs

Polarography depends on the current-voltage changes arising at a microelectrode when diffusion is the rate-limiting step in the discharge of ions. Both qualitative and quantitative analyses are possible if the substance is capable of undergoing cathodic reduction or anodic oxidation.

The commonest form of polarograph is the dropping mercury electrode. The cathode is mercury dropping from a glass capillary at a constant rate of about one drop every 3 seconds. The anode is a pool of mercury at the bottom of the vessel. A varying potential is applied and the current-voltage curve recorded. Only a very small residual current will flow until the applied voltage exceeds the decomposition potential of the substance present. It then rises rapidly to a plateau (the limiting current) corresponding to the maximum rate at which the ion species can be discharged. This is determined by the rate of diffusion of the ion to the electrode and is proportional to its concentration in the bulk solution. The material is characterized by its half-wave potential —that is, the potential at the point of inflexion of the current-voltage curve. The solution must be at constant temperature and quiescent. For quantitative work, the method is standardized either by direct comparison with a standard solution, by standard addition or with some suitable internal standard.

The method employs relatively simple instruments, preferably with automatic recording, and requires only small volumes of dilute (about 10^{-5} mol/l) solutions. Its sensitivity can be increased by plotting derivative curves. Although the dropping mercury electrode is the most versatile type of polarograph, rotating noble-metal electrodes such as platinum have been used for special applications (W18). Although the method has an extensive literature, it has not found wide acceptance in clinical chemistry, perhaps because of the difficulty of interpreting chart recordings obtained with complex biological solutions. It has been extensively used to study proteins, particularly sulfhydryl and disulfide groups (H20). Some compounds that are not polarographically active

can be studied after formation of an appropriate derivative. The method may also find an application in the analysis of free and protein-bound drugs, since the latter diffuse more slowly.

12.3. Coulometers

Coulometric methods are based on the measurement of the quantity of electricity (in coulombs) passing through a cell during an electrochemical reaction. The substance present is oxidized or reduced at one of the electrodes or reacted with a reagent generated by electrolysis. Equipment may either use a controlled potential at the working electrode (potentiostatic coulometry) or a constant current (amperostatic coulometry). Controlled-potential coulometers contain four units—a coulometer, a dc current supply, a potentiostat, and an electrolyte cell. The equipment is expensive and requires relatively long electrolysis times. Constant current procedures require only a stable current supply (1–200 mA) and an accurate timing device; they are cheaper and more widely used (W18).

Commercial instruments for the coulometric titration of chloride contain two silver electrodes and Ag ions are generated at a constant rate by a constant current coulometric circuit. The amperometric or indicator current remains constant until nearly all Cl ions are precipitated as silver chloride. The end point is indicated by a sudden increase in amperometric current due to excess Ag ions, and the result is given by the quantity of electricity (coulombs = amperes × time) passed (C21). Purdy (P14) has discussed the theory of coulometric titrations in detail, with particular reference to titrants, end point detection, sensitivity, precision, and accuracy, and has described a series of ingenious methods for use with biological fluids. Glucose is determined by reacting with glucose oxidase to form hydrogen peroxide. This reacts with KI in the presence of a molybdenum catalyst and the resulting iodine absorbed by thiosulfate. The excess thiosulfate is determined by titration with coulometrically-generated iodine to a dead-stop end point (S12). A similar principle is used to determine uric acid by titrating total reducing substances with coulometrically generated iodine before and after treatment with uricase (T12).

12.4. Other Methods

A wide variety of multipurpose electroanalytical instruments are available, several of which include provision for both controlled current and controlled potential operation (B1). Automatic titrators record the potential against the volume of titrant, and in some a greater accuracy is possible by using derivative curves obtained with an associated circuit.

Measurement of capacitance has been used to determine fecal fat, but as the capacitance of medium and long-chain triglycerides differs, calibration is a problem (H2).

In general, electrometric methods have found little application in clinical chemistry, but some of these may become more popular in future, particularly in view of their cheapness and the ease of recording the electrical signal.

13. Conclusions

During recent years the cost of instruments used in clinical chemistry has greatly increased, and much is now so expensive that it cannot be provided in every independent laboratory, particularly if it is used for only 35–40 hours per week. With modern instrumentation and technology, virtually any analysis becomes feasible—at a price—and the cost-benefit of all investigations is likely to be increasingly scrutinized. It is therefore imperative to consider first what information is required before selecting an analytical method and choosing an appropriate instrument.

The work of the clinical chemistry laboratory can be grouped into three areas, each of which requires different instrumentation: (1) Multicomponent analysis linked to a computer programmed to give diagnostic information, (2) single analyses providing specific information, and (3) emergency tests and progress-monitoring of patients on a 24-hour basis. Mitchell and Goldberg (M16) suggested that an effective viable laboratory required at least 8 sections, each specializing in one branch of clinical chemistry. Centralization of work into specialist units, each with an expert and suitable instrumentation, would improve the service and, by using staff and equipment more efficiently, reduce costs. However, a specialized instrument can often be used in many different branches of the subject. For example, column chromatography, often linked with a mass spectrometer, is used for steroid analysis, toxicology and metabolic studies; radioimmunoassay for specific proteins, peptide hormones and, increasingly, steroid and enzyme analysis; automatic methods are applied to many different types of molecule, and the computer can serve all instruments. Duplication of equipment can therefore be minimized only if expensive instruments form the focal points of activity. Although large centralized laboratories can be more efficient in economic terms, the small laboratory will still be needed for tests which must be made close to the patient. For a group of neighboring hospitals, these needs could be met most economically by a network of laboratories, where each would provide an emergency and monitoring service for its own hospital plus a specialist service for the group (M16).

Despite rapid advances in instrumentation, it is doubtful whether any

is yet good enough, owing, in part, to the difficulty of defining in quantitative terms the required performance and cost. However, as with automobiles this year's model is usually better than last year's, although probably more expensive. Improved reliability of instruments is necessary, but not all manufacturers realize the urgency needed to deal with equipment failure in a clinical laboratory. A few guarantee to maintain the performance of their instruments or systems at a satisfactory level and, although this service is expensive, it is probably the only method of ensuring that the work of the laboratory, and ultimately the patient, does not suffer. "Standby" instruments and within-laboratory maintenance facilities can be used to buffer the effect of an instrument breakdown.

Many automatic systems can analyze specimens faster, cheaper, and more precisely than a human analyst. Major areas of development will be in multichannel analysis and data processing, including computers. Since engineering, electronics, and computer specialists are being increasingly employed in the clinical chemistry laboratory, what is the future role of the analyst? Is he becoming subservient to machines? Although an instrument may determine the feasibility of the analysis, the clinical chemist must know whether it is worth doing, ensure that it is performed satisfactorily and be able to interpret the result. Inside most large analytical machines a chemical reaction is taking place. Automation should provide him with the time to investigate new areas and, where necessary, initiate the development of new instruments. Used properly, instruments can improve the quality, productivity and range of the work of the clinical chemist, and his education cannot be considered complete unless it includes training in instrumentation. While a knowledge of electronics is useful, an understanding of the physical principles and limitations of instrumental analysis is more important (S15). Uncritical users of black boxes pay for their lack of understanding by failure to exploit the performance of an instrument and to detect its errors (W8).

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